

Harnessing Mechanisms of Immune Modulation by Sorafenib to Augment
the Efficacy of Cellular Immunotherapy

by

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Abstract:

The tumor microenvironment is established and maintained through the complex interactions of tumor cells with host stromal elements. Therefore, therapies that target multiple cellular components of the tumor may be most effective. Sorafenib, a multi-kinase inhibitor, alters signaling pathways in tumor cells and host stromal cells. Thus, we explored the potential immune-modulating effects of Sorafenib in a murine HER-2-(neu) overexpressing breast tumor model alone and in combination with a HER-2 targeted granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting vaccine. In vitro, Sorafenib inhibited the growth of HER-2 overexpressing NT2.5 tumor cells, inducing apoptosis. Western blot analysis revealed that Sorafenib interfered with ERK MAPK, p38 MAPK, and STAT3 signaling, but not HER-2 or Akt signaling. It also decreased D-type cyclin expression. In vivo, single agent Sorafenib disrupted the tumor-associated vasculature and induced tumor apoptosis, effectively inducing the regression of established NT2.5 tumors in immune competent FVB/N mice. Immune depletion studies demonstrated that tumor rejection was mediated by both CD4⁺ and CD8⁺ T cells. Sorafenib treatment enhanced tumor clearance induced by vaccination with a GM-CSF-secreting, HER-2-expressing cellular vaccine in tumor-bearing FVB/N mice relative to either drug treatment or vaccination alone. Although the magnitude of the peripheral antigen-specific T cell response was unchanged, Sorafenib appeared to enhance antigen-specific T cell accumulation at the tumor site. Overall, these findings suggest that dendritic cell-based immunotherapy can be integrated with Sorafenib, resulting in enhanced therapeutic response

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Chapter 1: Introduction

HER2

The human epidermal growth factor receptor 2 (HER2, also known as *c-erbB-2*, or HER2/*neu*) is a member of HER family of transmembrane receptor tyrosine kinases. The HER family is comprised of four homologous epidermal growth factor receptors: HER1(EGFR/*erb1*), HER2 (*erb2*), HER3 (*erb3*), and HER4 (*erb4*). These receptors are involved in regulating cell growth, differentiation, and survival through signaling via PI3K/Akt and Ras/Raf/MEK/MAPK pathways^{1, 2}. While there are many ligands that have been identified that can activate individual HER receptors, no ligand has yet been identified for HER2. Upon ligand binding, HER receptors form homodimers and heterodimers with other members of the HER family, of which HER2 is the preferential dimerization partner. The heterodimerization between HER2 and the other HER receptors in the family allow its participation in signal transduction in the absence of a ligand. Heterodimers involving HER2 seem to show particularly high signaling potency compared to other dimerization combinations within the HER2 family³.

In vitro and animal studies have indicated that HER2 gene amplification and protein overexpression plays an essential role in oncogenic transformation, tumorigenesis, and metastasis⁴⁻⁶. Normal epithelial cells possess two copies of the HER2 gene and expresses low levels of HER2 protein on the cell surface. With oncogenic transformation, HER2 gene amplification generates more than two gene copies and increased mRNA transcription, which results in 10-100 fold increases in HER2 homodimer formation on the cell surface. Therefore, overexpression of the HER2 protein leads to constitutive activation

of downstream signaling pathways, which ultimately results in oncogenic transformation of cells to cause cancer⁷.

HER2+ breast cancer

Breast cancer is currently the most common cancer in women and is leading cause of cancer death in women in Western countries after lung cancer. Amplification of the HER2 gene in addition to overexpression at the messenger RNA or protein levels occur in about 20% of invasive breast cancers and corresponds to more aggressive disease and poor prognosis⁸. HER2 status has been shown to be a predictive marker of therapeutic response to HER2-targeted therapy⁹. Also, the accessibility of HER2 on the cell surface makes it a druggable target.

HER2-targeted treatments

Standard therapy for breast cancer includes surgery, radiation therapy, chemotherapy and endocrine therapy¹⁰. Optimal integration of these therapies has led to improvements in clinical outcome for breast cancer patients. More recently, some targeted therapies have improved overall survival for those women affected with metastatic disease. In this category, HER2+ breast cancer patients have seen small overall survival benefit with the development and FDA-approval of therapies available that target HER2. Trastuzumab (Herceptin) is a HER2-specific monoclonal antibody that binds to the extracellular domain of the HER2 protein. Lapatinib (Tykerb) is a dual HER2/EGFR1 tyrosine kinase inhibitor. Pertuzumab (Perjeta) is a monoclonal antibody that binds to the surface HER2 and works by inhibiting receptor dimerization and downstream signaling potential. Trastuzumab

emtansine (T-DMI, Kadcyla) is the HER2-specific monoclonal antibody, Trastuzumab, conjugated to cytotoxic molecules¹¹.

The development of these new therapies has improved clinical outcome for patients with HER2-positive breast cancer. However, relapse still occurs with current therapies, reflecting acquired resistance in some patients. Additionally, patients with metastatic cancer still eventually progress in their disease and metastatic breast cancer remains incurable. The limitations of current therapies lie in the common toxicities of treatments to both malignant and normal tissues and the occurrence of relapse due to outgrowth of resistant cancer cells. Therefore, the ability to successfully combat the disease will rely heavily on the development of unique targeted therapies with distinct mechanisms of action that preferably impact malignant tissue.

Immunotherapy for Cancer Treatment

Immunotherapy provides an attractive option to overcome these distinct resistance mechanisms through the utilization the patient's own immune system to combat their disease. Additionally, immunotherapy allows a mechanism for targeting the malignant cells specifically while leaving normal cells unharmed. Using immunotherapy as a means of treating cancer dates back to 1891, when William B. Coley found that killed bacteria injected into bone sarcoma resulted in reduced tumor size. Similar crude bacterial mixtures, called "Coley's toxins," were used to treat a variety of different cancers from 1893 to 1963 with varying clinical benefit¹². Further data to support immunotherapy came from clinical trials carried out in the 1980's in metastatic melanoma and renal cell carcinoma. In these trials, patients were treated with interleukin-2 (IL-2). Uniquely, it was

known that IL-2 would have no direct cytotoxic effects on the tumor. Instead it was used specifically to stimulate the proliferation of cytotoxic T cells (CTLs) ¹³. In these studies 15% of patients showed response and about half of the responsive patients were cured of their disease. This led to the approval of IL-2 by the FDA in the 1990's as the first immunotherapy to treat cancer. More recently, immunotherapy has gained momentum with the FDA approval of sipuleucel-T (Provenge), a dendritic cell based vaccine, for the treatment of prostate cancer; and ipilimumab (Yervoy), a monoclonal antibody against cytotoxic T lymphocyte antigen-4 (CTLA-4), and Pembrolizumab (Keytruda), a monoclonal antibody specific for PD-1 (programmed cell death-1). These two monoclonal antibodies are approved for the treatment of metastatic melanoma ^{14,15}.

Currently, there is little doubt of immune system's role both in cancer development and successful disease eradication. As we have gained a deeper understanding of the complex molecular and cellular mechanisms that comprise the immune system, we have subsequently enhanced the development of new therapies to induce and manipulate the anti-tumor immune response. One of these immunotherapeutic approaches has been cancer vaccines.

Cancer Vaccines

Successful therapeutic cancer vaccines will result in both primary activation of the immune system to recognize and attack cancer cells within the host, and the development of secondary immunological memory that prevents reoccurrence. In order to accomplish this, cancer vaccines consist of an immunogenic tumor antigen to stimulate the activation of

helper T cells and CTLs that can recognize tumor cells and initiate tumor cell destruction mechanisms¹⁶.

Because it remains unclear what the most potent tumor antigens are, one approach has been to use whole cells for vaccination. Early generation cancer vaccines took the form of killed tumor cells or tumor cell lysates mixed with bacteria adjuvants in an attempt to amplify anti-tumor immunity^{17, 18}. Next generation vaccines replaced the crude bacteria-lysate mixtures with genetically modified tumor vaccines. In the 1960's, Lindermann and Klien were able to show that tumor cells infected with influenza virus were able to generate enhanced tumor cell immunogenicity¹⁹. Cells have also been transduced with viral genes or allogeneic MHC genes in an attempt to enhance systemic immune responses^{20, 21}. Another class of genetically modified cell-based vaccines takes advantage of the ability of cytokines and co-stimulatory molecules constitutively expressed on the vaccine cells to activate local inflammatory response, sparing systemic toxicity. Specifically, granulocyte-macrophage colony stimulating factor (GM-CSF) has been shown to be most potent in its ability to modify tumor immunogenicity²².

The activity of GM-CSF modified vaccines lies in their effectiveness at promoting the activation and maturation of dendritic cells (DCs) at the vaccine site. DCs are central to activation of naïve T cells in peripheral lymphoid tissues to mount a successful immune response. A large number of clinical trials in a variety of cancers have proven the efficacy of GM-CSF transduced vaccines to boost patient's anti-tumor immune responses²³⁻²⁶. These trials also pointed to an important role of conventional therapies, such as chemotherapy, to have an effect on vaccine-induced immune responses. This was clearly demonstrated in patients with metastatic breast cancer, where GM-CSF-secreting whole

cell vaccination in the presence of immune-modulating doses of cyclophosphamide and doxorubicin enhanced HER2-specific antibodies and HER2-specific DTH responses²⁷.

In order to effectively implement cancer vaccines in the clinic, it is necessary to have a basic understanding of the anti-tumor immune response and the subsequent dysregulation that can occur in cancer patients. Successful cancer vaccination therapies must reprogram the immune response to actively target cancer cells and simultaneously relieve suppressive mechanisms that can hinder productive anti-tumor immune responses.

The anti-tumor immune response- T cell activation and Antigen Presenting Cells

The immune response mounted against a tumor relies on both the innate and adaptive arms of the immune system. Cells in the innate immune system are not antigen specific. Instead, innate immune cells actively survey the host and recognize cell-surface stress-associated and danger-associated molecular patterns. For example, natural killer (NK) cells can recognize “non-self” or “stressed self” cells in the host²⁸. Additionally, antigen presenting cells (APCs), such as DCs present in the periphery, can recognize danger signals through interaction of cell surface receptors. These danger signals include: Toll receptor or NOD-like receptors (NLRs) ligation; retinoic acid-inducible gene 1 (RIG1) sensing of RNA; or stimulator of interferon genes (STING) pathway activation as a result of cytosolic DNA recognition^{29,30}. Upon activation, DCs undergo maturation to upregulate co-stimulatory molecules and act as messengers to relay the danger signals to secondary lymphoid tissues where they stimulate the activation of the adaptive arm of the immune response.

The adaptive immune response, unlike innate immunity, is antigen specific. There are two arms to adaptive immune responses, humoral immunity and cell-mediated immunity. The

humoral response is dependent on B cells and ultimately leads to the production of antibodies. Cell-mediated immune responses depend on T cells. Whereas B cells only respond to intact antigen and thus recognize extracellular antigenic epitopes to mount a response, T cells are able to respond to both extracellular and intracellular proteins. T cell-mediated immune responses require multiple steps including: the clonal selection of antigen-specific cells, activation and proliferation of the selected cells in secondary lymphoid tissues, subsequent trafficking to the tumor site, and lastly, the ability to execute their specific effector functions once within the tumor³¹.

There are two major functionally different types of T cells defined by the cell surface expression of distinct co-receptors proteins: CD4⁺ helper T cells and CD8⁺ cytotoxic T cells. As their name implies, cytotoxic CD8⁺ T cells are able to kill target cells directly whereas CD4⁺ helper T cells activate APCs and provide “help” to enhance CD8⁺ T cell activation. CD4⁺ T cells also provide help to B cells to stimulate antibody production. A major differentiating factor between CD4⁺ and CD8⁺ T cells is the context by which their T cell receptors recognize and bind to antigenic peptides on histocompatibility complex (MHC) molecules. There are two types of MHC molecules, MHC class I and MHC class II, which differ in their structure and expression levels within the body. MHC class I molecules are expressed on all nucleated cells in the body and present intracellular peptides to CD8⁺ T cells. The proteasome pathway within the cell processes proteins and cleaves them into peptide fragments ranging between 8-12 amino acids in length. These peptide fragments are loaded on the MHC class I molecules for presentation to CD8⁺ T cells^{32, 33}.

MHC class II molecules are expressed only on a specialized subset of APCs, including B cells, DCs, and macrophages. APCs take up exogenous proteins (or extracellular microbes)

and process these proteins through the lysosomal degradation pathway. This pathway results in protein processing into peptides ranging from 10-25 amino acids in length ^{32, 34}. These peptides are loaded onto MHC class II molecules and presented on the surface of APCs to activate CD4+ T helper cells.

During development, T cells undergo a selection process in the thymus. Positive selection, also known as MHC restriction, ensures only those T cells expressing T cell receptors (TCRs) that recognize and bind self-MHC molecules are allowed to survive. Thymic stromal cells are responsible for mediating positive selection. CD4+ or CD8+ cell fate is determined by the specificity of the TCR to recognize and bind invariant sites on either MHC class I or MHC class II ³⁵, as binding of both the TCR and a single co-receptor is necessary to promote T cell survival. The cellular signals that promote one cell lineage over the other seems to depend, at least in part, through differential leukocyte-specific tyrosine kinase (Lck) signals upon engagement of the TCR receptor with either co-receptor.

Additionally, T cells also undergo negative selection which eliminates T cells with very high avidity for self-MHC/peptide complexes. The process of negative selection is mediated primarily by APCs in the thymus, such as DCs and macrophages. T cells that react too strongly with self-antigen are induced to die by apoptosis. Under normal circumstances, this prevents the maturation of T cells that would attack the host's own cells, thereby avoiding autoimmunity³⁵.

T cells that have survived selection in the thymus are then carried in the blood to peripheral lymphoid tissues to interact with their specific antigens and undergo proliferation. T cells require two signals for activation. Signal 1 is the result of the interaction of an antigenic

peptide with the TCR-CD3 complex³⁶. The CD3 complex is associated with the TCR and is required for proper TCR expression and signal transduction upon activation. The CD3 complex is composed of the molecules CD3 ϵ , CD3 γ , and CD3 δ in addition to a ζ chain, which is a disulfide-linked homodimer³⁷. Antigen recognition in the context of peptide:MHC initiates tyrosine phosphorylation of immune-receptor tyrosine-based activation motifs (ITAMs) on the intracellular regions of the CD3 complex and the ζ chain by the Src kinase Lck³⁸. These phosphorylated ITAMs provide a docking point for the recruitment of Syk family kinase, Zeta-activated protein 70kDa (Zap70). Zap70 then phosphorylates the protein linker for the activation of T cells (LAT), recruiting SLP76, which complexes with LAT proteins after phosphorylation by Zap70³⁹. This LAT-SLP76 interaction provides a docking site for several signaling effectors through binding to the phosphotyrosine binding sites. One of these effectors, phospholipase C- γ , transduces signals resulting in activation of Ras and mitogen-activating protein kinase (MAPK) as well as the influx of calcium into the cytosol. This signaling results in the activation of transcription factors Fos and Jun that form the AP-1 complex, the translocation of nuclear factor of activated T cells (NFAT), and nuclear factor- κ B (NF- κ B). These three factors act together to activate the transcription of interleukin-2 (IL-2) gene⁴⁰.

Engagement of the TCR-CD3 complex alone is insufficient for T cell activation. A second signal is required to achieve optimal T cell activation and proliferation. The principal “second signal” is provided by interactions between the CD28 molecule on T cells and B7 proteins on APCs⁴¹. Ligands for B7 are CD28 and CTLA-4 (CD 152), which act antagonistically with each other. Signaling through CD28 and B7 leads to the phosphorylation of Src-family resulting in the recruitment of several downstream proteins,

including Grb2, Vav, and ITK that ultimately augment IL-2 production and activate T cell proliferation. Conversely, engagement of CTLA-4 attenuates T cell proliferation signals⁴⁰.

T cell tolerance

T cell tolerance, which is the ability of the immune cells to differentiate self from non-self, is the foundation of a healthy functioning immune response. As mentioned previously, this prevents reacting to self-antigens and resulting autoimmunity. However, these self-protective mechanisms also provide the biggest challenge for successful cancer vaccines. As tumors arise from altered “self” cells; an inadequate immune response to “self” permits tumor growth. Therefore, successful vaccination requires breaking immune tolerance to recognize and attack host tumor cells.

T cell tolerance is maintained at two levels, central and peripheral tolerance. Central tolerance occurs by deletion of self-reactive T cells in the thymus. As described earlier, thymocytes expressing TCRs that have high-avidity for self-peptide-MHC are induced to undergo apoptosis, thus preventing potentially self-reactive T cells from entering the circulation⁴². As all potential self-antigens are not expressed in the thymus, peripheral tolerance mechanisms come into play to inhibit circulating self-reactive T cells. Three major mechanisms of peripheral tolerance include: deletion, ignorance, and anergy.

Deletion of self-reactive T cells in the periphery occurs by a mechanism similar to that in the thymus- induction of apoptosis. Both Bim signaling and Fas-mediated death receptor signaling cooperate in tandem to ensure killing of T cells that respond too strongly to self-antigens in the circulation. Fas (CD95) is a death-domain-containing receptor that is activated by binding to its corresponding ligand FasL (CD95L). The activation of the Fas

receptor on T cells by cells containing FasL, induce both the up-regulation of FasL on T cells themselves as well as that activation of an intracellular death-inducing signaling complex (DISC). DISC activates caspases to promote apoptosis by activation-induced cell death^{43,44}. Concurrently, Bim activates Bax/Bak, which causes mitochondrial permeabilization to induce apoptosis⁴⁵.

Ignorance occurs as a result of low level antigen expression or antigen sequestration, which results in T cells that remain naïve due to lack of antigen exposure⁴⁶. T cell activation in the absence of a second signal results in hyporesponsiveness, termed “anergy.” Anergy results in repression of TCR signaling and decreased IL-2 expression⁴². Additionally, inhibitory signaling molecules can be engaged as a second signal on T cells. One such example is programmed cell death 1 (PD-1) and its ligands PD-L1 and PD-L2. PD-1 association with its ligand results in PD-1 ligation with the TCR. This ligation activates phosphatases that attenuate T cell proliferation pathways⁴⁷. In this way, PD-1 interactions can limit the expansion of self-reactive T cells. PD-1 signaling can be manipulated by the tumor to prevent expansion of tumor-reactive T cells as well⁴⁸.

Regulatory T cells

CD4⁺ T regulatory cells (Tregs) are produced in the thymus, forming a functionally distinct T-cell subpopulation in the periphery. A distinguishing feature of Tregs is their expression of the transcription factor, forkhead box p3 (FoxP3)⁴⁹. FoxP3 controls the expression of several characteristic genes for cell surface molecules, such as the alpha chain of the IL-2 receptor, CD25, glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR) family regulated gene and CTLA-4, which are also highly expressed in conventional T

cells after TCR stimulation^{50,51}. FoxP3 inhibits TCR-activation-dependent production of effector cytokines including IL-2 and IFN- γ . As possible mechanisms of suppression, it has been shown that FoxP3+ Tregs exert suppression by cell-to-cell contact with APCs, such as DCs. FoxP3+ Tregs are also able to secrete immunosuppressive cytokines such as interleukin 10 (IL-10), transforming growth factor β (TGF- β) and interleukin 35 (IL-35)⁵². In this way, Tregs are capable of suppressing a wide variety of immune responses against self-antigens, including tumor antigens.

Immune System Evasion- Immunoregulatory Components of the Tumor Microenvironment

The host antitumor immune response can sculpt tumor growth, invasion, and metastasis in a variety of ways. The prevention of immune cell access into the tumor, the accumulation of inhibitory Tregs and/or other suppressive cells, the activation of negative immunoregulatory pathways, and the dysregulation of effector T cells are all mechanisms by which tumors evade the host immune system.

Notably, the presence of large numbers of tumor infiltrating T lymphocytes (TILs) has been reported to be an indicator of good prognosis in multiple solid tumors⁵³⁻⁵⁶. Therefore, it is not surprising that physically preventing effector CD8+ T cell infiltration or inhibiting their activity once they gain access to the tumor might be a means by which tumors protect themselves from immune attack, enabling them to persist within the host. Additionally, distinct components of the tumor microenvironment can suppress active antitumor T cell responses in multiple ways. Tumor endothelial cells (TECs) present at the blood-tumor barrier act as gatekeepers, regulating the homing, adhesion and trans-endothelial migration

of lymphocytes into the tumor⁵⁷. TECs can create a protective barrier to block or disrupt trans-endothelial T cell migration and survival within the tumor microenvironment. Many TECs express FasL and induce the death of Fas-expressing T cells attempting to gain access to the tumor⁵⁷.

Additionally, both innate and adaptive immune cells that gain access to the tumor site can contribute to disease progression by corruption of the inherent protective inflammatory response mounted against the tumor to promote immune evasion. For example, alterations in tumor cell biology can lead to decreased susceptibility to killing, and alterations in APCs can lead to faulty T cell priming and promote T cell dysfunction. Both the induction of suppressive cytokines and the expression of negative immunomodulatory molecules within the tumor microenvironment can dampen immune responses. High levels of IL-10 and/or transforming growth factor β (TGF- β), the expression of FAS or FASL, PDL-1 PDL-2, and the expression of immunomodulatory enzymes like indoleamine 2,3-dioxygenase, (IDO), arginase (ARG) or inducible nitric-oxide synthase (iNOS) can inhibit tumor immunity⁵⁸. The major producers of these immunoregulatory molecules include tolerogenic DCs, Tregs, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs).

Of these suppressive cell types, breast cancer is characterized by having a large population of TAMs, and experimental models have shown multiple pathways by which TAMs can influence the surrounding tumor microenvironment⁵⁹. TAMs have been shown to secrete pro-angiogenic factors, such as VEGF, that support the development of neo-vasculature paramount to tumor survival and metastases to distant sites. Additionally, TAMs can

secrete cytokines and other factors that can suppress the induction of local pro-inflammatory antitumor response⁶⁰.

Vaccine strategies to reprogram the immune response to cancer

Despite the many immunosuppressive mechanisms that blunt productive anti-tumor responses, it is clear that the presence of immune cell infiltrates are associated with improved survival and response to therapy in some patients. These observations imply that the tumor microenvironment represents a therapeutic target that can be manipulated to promote tumor regression in more patients. Therefore, preclinical work has aimed to integrate tumor vaccines with established cancer drugs in an effort to target cancer cells directly through cytotoxic effects, as well as potentially augmenting vaccine-induced immune responses through modulating immune cells within the tumor microenvironment.

A Preclinical Model of Antigen-Specific Immune Tolerance

The *neu*-N transgenic mouse was derived from parental FVB/N mice by placing the rat *neu* proto-oncogene under the control of the mammary specific promoter, mouse mammary tumor virus (MMTV), resulting in mammary tissue specific expression of the rat HER-2 protein. As a result of overexpressing HER-2, *neu*-N mice spontaneously develop mammary tumors at about 4-6 months⁶¹. These tumors were used to develop cell lines that express high levels of rat HER-2, called NT2.5. These cell lines are used for orthotopic tumor implants to examine HER2 responses in parental FVB/N where rat HER-2 is immunogenic, and in *neu*-N mice where it is not due to immune tolerance.

A whole cell vaccine was created from 3T3 fibroblast cells genetically modified to constitutively secrete GM-CSF and deliver high amounts of tumor antigen through the

overexpression of rat HER-2⁶². Tumors were orthotopically implanted into the mammary fat pads of FVB/N mice and allowed to reach ~0.5 centimeter in size (about 1 week following implant). Vaccination of these mice with the HER-2 overexpressing GM-CSF-secreting 3T3 vaccine cells inhibited tumor cell growth and ultimately resulted in 100% tumor resolution in these mice. Evaluation of specific anti-tumor responses in these mice showed that FVB/N mice develop high levels of antibodies that are specific for HER-2 in addition to a population of high avidity T cells that are specific for the immunodominant epitope of rat HER-2, RNEU₄₂₀₋₄₂₉ also called, p50^{63, 64}.

To this end, previous successful animal and human studies have examined combining vaccination with chemotherapy and Trastuzumab^{27,62,65-68}. These combinations were shown to enhance vaccine induced immune responses, through measuring both HER-2 specific antibody production and HER-2 specific T cell responses. Studies in these models have led to clinical trials that have examined the use of a human vaccine in the clinic for patients with HER-2 positive as well as HER-2 negative disease and have seen some success²⁷.

These preclinical studies were also expanded to explore the potential use of angiogenesis inhibitors in combination with vaccine. Despite many efforts to incorporate anti-angiogenic therapy into a treatment standard for breast cancer, they have not been successful. Therefore, antiangiogenic therapy may work best in combination therapy rather than as single agents⁶⁹. Previous published work focused on the immune based activity of DC101, a monoclonal antibody that targets vascular endothelial growth factor receptor 2 (VEGFR-2). VEGFR-2 is found on endothelial cells and has been shown to play a critical role in initiating the formation of new vessels that is hallmark of cancer development.

Treating tumor-bearing FVB/N mice with DC101 resulted in tumor regression when compared with IgG controls. Tumor resolution was accompanied by increased HER-2 specific T cells even in the absence of vaccination. T cell depletion studies in mice treated with DC101 demonstrated a dependence on both CD4⁺ and CD8⁺ T cells for drug efficacy⁷⁰. Giving DC101 sequenced with HER-2 targeted GM-CSF vaccination resulted in both further enhancement of tumor resolution compared to either single therapy agent, and enhanced T cell responses against the tumor, specifically, against the immunodominant epitope of HER2.

Given the problem of development of acquired resistance with many VEGF- targeted therapies, multi-tyrosine kinase inhibitors (TKIs) are an attractive option to target angiogenesis given their ability to concurrently target other compensatory pathways important in the growth and development of cancer cells. One such TKI, Sorafenib (Nexavar) is a multiple serine/threonine kinase inhibitor that was originally designed to inhibit Ras kinase activity but was later shown to have significant activity against several other receptor tyrosine kinases involved in neovascularization and tumor progression, VEGFR-2, VEGFR-3, platelet-derived growth factor (PDGFR)- β , Flt-3, and c-KIT ⁷¹. Sorafenib has been approved for the treatment of renal cell carcinoma (RCC), hepatocellular carcinoma (HCC) and more recently for the treatment of differentiated thyroid cancer (RTC)⁷²⁻⁷⁴.

Objectives:

The hypothesis of this thesis was Sorafenib modulates immune cells within the tumor microenvironment to enhance tumor rejection and support the anti-tumor immune response

to improve the efficacy of DC-based, HER-2 targeted, GM-CSF-secreting vaccination. This was investigated first by examining the effect of single agent Sorafenib on immune cells within the breast tumor microenvironment. The HER2-overexpressing cell line, NT2.5, was used to analyze the effect of Sorafenib on HER-2 over-expressing breast cancer cells both *in vitro* and *in vivo*. Given the reported potential immune modulating effects of TKIs on cells within the tumor microenvironment, the interaction of Sorafenib with immune cells was determined. Specifically, the effect of Sorafenib on T cells and macrophages was analyzed. Finally, the therapeutic and immune effects of partnering Sorafenib with DC-based vaccination were investigated. These studies support the hypothesis that Sorafenib can be successfully re-purposed as a partner for immunotherapy, not only by inducing increased cell death and inhibiting angiogenesis, but by acting through an immune-based mechanism to accelerate tumor clearance.

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Chapter 2: Characterizing the Mechanism of Therapeutic Activity of Sorafenib in HER2+ Breast Cancer in FVB/N mice

Introduction:

The tumor microenvironment is established and maintained through the complex interactions of tumors cells with host stromal elements. Therefore, multi-targeted drugs and combination therapies that target multiple cellular components of the microenvironment may be the most effective strategy to improve survival in patients with breast cancer. One principle targetable component of the tumor microenvironment is the vascular niche, where angiogenesis occurs.

Angiogenesis is defined as the development of a neo-vasculature from pre-existing blood vessels. Angiogenesis is now recognized as a hallmark of cancer development since the ability of cancer cells to acquire new blood vessels is paramount to support tumor cell proliferation and growth by providing necessary oxygen and nutrients to the tumor site¹. Angiogenesis is also necessary for the metastasis of cancer cells to distant sites. Beginning in the 1970's when Judah Folkman first recognized that tumor growth is dependent on angiogenesis, significant investments have been made in the development of anti-angiogenic therapy for the treatment of cancer in the clinic². As a result of this research, inhibitors of angiogenesis have been developed.

In cancer development, VEGF signaling is a major player in the “angiogenic switch” which is the rapid increase in blood vessel formation to support tumor growth and development when tumors reach a size beyond 2mm²³. Therefore, one strategy to target angiogenesis is through the use of therapies that target various aspects of VEGF signaling. Most notable

is the first clinically approved inhibitor of angiogenesis, Bevacizumab (Avastin). Bevacizumab, a humanized monoclonal antibody, works mainly by binding to the biologically active forms of VEGF thereby preventing its interactions with VEGF receptors⁴. Despite a modest increase in progression free survival with the use of single agent Bevacizumab, many patients do ultimately progress due to therapeutic resistance^{5,6}.

Given the problem of acquired resistance to anti-VEGF therapy, TKIs are an attractive option to target angiogenesis in their ability to also target other compensatory pathways important in the growth and development of cancer cells^{7,8}. Notably, targeting the immune system has been shown to play a role in the antitumor effect of many conventional cancer therapies, including angiogenesis inhibitors, such as TKIs⁹.

Previously, the impact of standard and novel cancer drugs on the immune system was explored¹⁰. It was reported that the VEGFR-2-specific monoclonal antibody DC101 not only disrupts the tumor-associated vasculature, but also promotes T cell-dependent, immune-mediated tumor rejection¹¹. These observations suggest that therapies targeting multiple cellular components of the tumor may be more effective than therapies that only target a single cellular element within the tumor. Several groups have investigated the immune-modulating effect of the TKI, Sunitinib, but less is known about the effects of Sorafenib on the immune system^{12,13}. Accordingly, these studies have been expanded to explore the immune-based activity of Sorafenib, a promiscuous small molecule kinase inhibitor that blocks signaling in both tumor cells and host endothelial cells¹⁴.

Sorafenib is a small molecular inhibitor of angiogenesis designed to inhibit RAF/MEK/ERK signaling, with a number of off- target effects including the inhibition of

wildtype and mutant BRAF, STAT3, and the receptor tyrosine kinases VEGFR2, VEGFR3, and PDGFR- β ¹⁴. It is FDA- approved for the treatment of HCC, RCC, and DTC¹⁵⁻¹⁷, and is under investigation in other tumor types as well. Sorafenib has been reported to support tumor immunity by decreasing the frequency of CD4⁺CD25⁺FoxP3⁺ Tregs without impacting the function of peripheral effector T cells in patients with RCC¹⁸. Conversely, Sorafenib has been shown to inhibit DC function, reducing DC maturation, migration, and T cell priming¹⁹. Most data support an inhibitory effect of Sorafenib on tumor-specific immunity¹⁹⁻²¹ but the variable immune effects of Sorafenib suggest they could be context-dependent.

Given the established clinical indications for Sorafenib, the increasing use of immunotherapy in the clinic, and the complex immune effects of Sorafenib, the immune-modulating effects of Sorafenib were investigated. First, the effect of Sorafenib on the growth characteristics and signaling pathways of HER-2-expressing NT2.5 mammary tumor cells *in vitro* was determined. *In vivo* tumor regression with Sorafenib treatment was examined. Finally, the effect of Sorafenib on the immune system was analyzed through depletion studies. The results of these studies identified a unique immune-based mechanism of Sorafenib to promote tumor cell clearance in FVB/N mice.

Materials and Methods:

Mice

FVB/N mice were purchased from Harlan (Frederick, MD) and 8 to 12 week old mice were used in experiments. Animals were housed in pathogen-free conditions and were treated in accordance with institutional and AAALAC policies. All protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

Reagents

Sorafenib was purchased from LC Laboratories (Woburn, MA). For *in vitro* studies, sorafenib was dissolved in dimethyl sulfoxide (DMSO) and further diluted in culture medium to the required concentration with the final concentration of DMSO concentration less than 0.2%. The p38 pathway inhibitor SB203580 was purchased from Sigma-Aldrich (St. Louis, MO). The ERK pathway inhibitor U0126 was purchased from Invitrogen (Carlsbad, CA). Antibodies for p-STAT3 (Tyr705), STAT3, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p38 (Thr180/Tyr182), p38, p-AKT (Ser473), AKT, p-HER2 (Tyr877), HER2, Cyclin D1, Cyclin D2, Cyclin D3, BCLXL, BCL2, and activated caspase 3 were all purchased from Cell Signaling Technologies (Beverly, MA). The actin antibody was purchased from Calbiochem (San Diego, CA). Rabbit anti-mouse PECAM/CD31 antibody was purchased from Abcam (Cambridge, MA). Clodronate liposomes were provided by Dr. Nico van Rooijen (Vrije Universiteit, VUMC, The Netherlands). The α -asialo GM1 antibody was purchased from Wako Chemical (Richmond, VA).

Cell Lines and Media

The NT2.5 tumor cell line, derived from a spontaneous tumor of a *neu*-N transgenic mouse, was grown as previously described²².

Cell Proliferation Assays

NT2.5 cells were placed in 96-well plates at 10^4 cells per well in complete growth media overnight. During drug treatments, media was replaced with media containing 0.5% FBS and 0 μ M-10 μ M Sorafenib in a final volume of 200 μ l. Final concentrations of DMSO were normalized within each experiment. At each time point, 100 μ l of media was removed and 20 μ l of CellTiter 96 Aqueous One Solution (Promega) was added for 2 hours at 37°C. Measurements were made at 2, 24, 48, and 72 hours at 490nm. Cell free wells containing media and CellTiter solution were used as blank controls.

Western Blotting

2×10^6 NT2.5 cells were placed in 6-well plates overnight in complete growth media. To analyze the effects of Sorafenib on HER-2, ERK, MAPK, p38 MAPK, STAT3 and AKT signaling, media was changed to media containing 0.5% FBS and incubated for 2 hours with 0 μ M-10 μ M of Sorafenib. To analyze cyclin expression, cells were incubated for 6-7 hours with 5 μ M and 10 μ M Sorafenib, U0126 (MEK/ERK inhibitor) or SB203580 (p38 inhibitor). After the incubation period, cells were lysed in ice-cold CellLytic cell lysis reagent (Sigma) supplemented with Phosphatase Inhibitor Cocktail 2 (Sigma) and EDTA-free protease inhibitor cocktail from Roche Diagnostics (Basel, Switzerland) for 5-10 minutes on ice. Cell lysates were scraped from 6-well plates, collected and centrifuged for 10 minutes at 10,000 RPM. Lysates were mixed 1:1 with Laemmli sample buffer and boiled for 8 minutes. Samples were subjected to SDS-PAGE on 4-15% gradient gels

(BioRad, Hercules, CA) and transferred to Amersham Hybond-ECL (GE Healthcare, Piscataway, NJ). Membranes were blocked for 1 hour in 5% Milk in TBS-Tween (w/v), and then incubated overnight with primary antibodies in 5% BSA in TBS-Tween (w/v) at the dilution recommended on the product data sheet. After washing, membranes were incubated with HRP-conjugated Goat- α -Rabbit IgG (Cell Signaling Technologies) for 30 minutes at room temperature, washed, and developed using HyGLO Quickspray (Denville Scientific, Metuchen, NJ). Membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions, then blocked and reprobed.

Immunohistochemical staining

Tumors were fixed in formalin for 24 hours, paraffin- embedded and sectioned at 5 μ m by the JHMI Pathology Core. Sections were stained with H&E or retained for immunohistochemistry at the JHMI Oncology Tissue Service Center. Vascularization and apoptosis were analyzed with antibodies specific for PECAM/CD31 (Cell Signaling) and cleaved caspase-3 (Abcam) respectively. Antigen retrieval was carried out for 45 minutes in HTTR steam (Target Retrieval Solution; Dako) followed by incubation with primary antibody for 45 minutes at room temperature. Slides were incubated with Power Vision Poly-HRP anti-rabbit IgG secondary antibody for 30 minutes at room temperature. Slides were developed with 3, 3' diaminobenzidine (Sigma Fast DAB tablets) and slides were counterstained with Mayers hematoxylin (Dako). Images were captured under light microscopy at 10x magnification (E600, Nikon). Three independent high-powered viewing fields were captured and staining was quantified using AR-Elements Microscope Imaging Software (Nikon).

Drug treatment

FVB/N mice were challenged subcutaneously with 5×10^6 NT2.5 tumor cells in the right mammary fat pad, followed by vaccination 10-14 days later. Sorafenib (30mg/kg) was administered in 100 μ l daily Monday through Friday by oral gavage with a feeding needle beginning the day of vaccination. A viscous vehicle composed of 30% (w/v) Cremophor EL, 30% (w/v) PEG 400, and 10% ethanol, 10% glucose (Sigma-Aldrich) was used both to dissolve Sorafenib and administered as the vehicle treatment control. Mice were monitored for tumor growth and onset twice weekly. Tumor growth was determined by measuring tumor diameter in two perpendicular dimensions with calipers. Mean tumor size for an experimental group included only those mice with measureable tumors.

Depletion Experiments

CD4⁺ and CD8⁺ T cells were continuously depleted using GK1.5 and 2.43 antibodies as previously described²². Natural killer cells were depleted by twice weekly intraperitoneal (i.p.) injections of α -asialo GM1 antibody. Macrophages were depleted by i.p. injection of clodronate liposomes weekly. Depletions were initiated one week prior to tumor challenge and maintained throughout the experiment.

Statistical Analysis

Statistical analysis was conducted either in Microsoft Excel or GraphPad Software using an unpaired, two-tailed Student's t-test, assuming equal population variances to determine the statistical significance between treatment groups. $P < 0.05$ was considered significant.

Results:

Sorafenib inhibits the growth of HER-2 over-expressing breast tumor cells *in vitro*

First, the effect of Sorafenib on the HER-2 over-expressing breast tumor cell line NT2.5 *in vitro* was examined. Sorafenib treatment inhibited NT2.5 cell growth, with a decrease in cell viability observed at concentrations between 1 to 10 μ M (Figure 1A). Flow cytometric analysis of Sorafenib treated NT2.5 cells stained with Annexin V and 7-AAD revealed a concentration-dependent increase in apoptosis (Figure 1B). The effect of Sorafenib on downstream targets of the HER-2 pathway was also investigated. Sorafenib interfered with ERK/MAPK, p38 MAPK, and STAT3 signaling, shown by decreased expression of the phosphorylated proteins at higher treatment concentrations. HER-2 or AKT signaling were not affected by Sorafenib treatment (Figure 1C). Sorafenib also decreased the expression of the G1/S cyclins D1, D2, and D3 in NT2.5 cells, whereas Bcl2 and BclXL expression were not affected (Figure 1D).

MAPK signaling is required for the expression of cyclin D1, whereas cyclin D3 can be controlled by additional pathways²³. Therefore, the effect of Sorafenib on these D-type cyclins relative to specific inhibitors of the ERK/MAPK and the p38 MAPK pathways was analyzed. Sorafenib inhibited cyclin D1 to a greater extent than either of the two specific MAPK pathway inhibitors, suggesting that the mechanism of NT2.5 growth inhibition by Sorafenib is dependent on both arms of the MAPK signaling pathway. However, unlike the either single arm MAPK inhibitors, Sorafenib also inhibited cyclin D3 (Figure 1D). Collectively, these data demonstrate that Sorafenib treatment induced apoptosis and inhibited cell growth of NT2.5 cells *in vitro* through MAPK-dependent and -independent mechanisms.

Sorafenib causes regression of HER-2 over-expressing breast tumors *in vivo*

The ability of Sorafenib to inhibit the growth of established NT2.5 tumors *in vivo* was then examined in immune competent FVB/N mice. Sorafenib monotherapy enhanced NT2.5 tumor regression in tumor-bearing FVB/N mice compared with vehicle-treated control mice (Figure 2A and B). Immunohistochemistry analyses of tumors harvested 12 days post-treatment showed that Sorafenib treatment increased the disruption of tumor-associated vasculature. Decreased endothelial cell-specific PECAM/CD31 staining was observed in tumors from Sorafenib treated mice compared to tumors from vehicle treated mice (Figure 2C). Sorafenib treatment also resulted in an increase in tumor cell death. Tumors from Sorafenib treated mice showed an increase in staining for cleaved caspase-3 compared to the tumors of mice receiving vehicle treatment (Figure 2D). Taken together, these data suggest that Sorafenib inhibits NT2.5 breast tumor cell growth by inhibiting angiogenesis and inducing apoptosis *in vivo*.

Sorafenib-mediated tumor clearance is T cell dependent

Studies selectively depleting distinct immune cells were conducted to evaluate the potential immune-dependent effects of Sorafenib. Selectively depleting either CD4⁺ or CD8⁺ T cells partially inhibited the efficacy of Sorafenib, whereas depleting both T cell subsets completely abrogated the anti-tumor effect of Sorafenib (Figure 3A and 3B). Depletion of NK cells or macrophages had no effect on the ability of Sorafenib to inhibit tumor growth (Figure 3C and 3D). In animals cured of their tumors by Sorafenib treatment, drug was withdrawn for one week and mice were re-challenged with 5×10^6 NT2.5 cells on the contralateral side. No new tumor development was observed at the secondary tumor

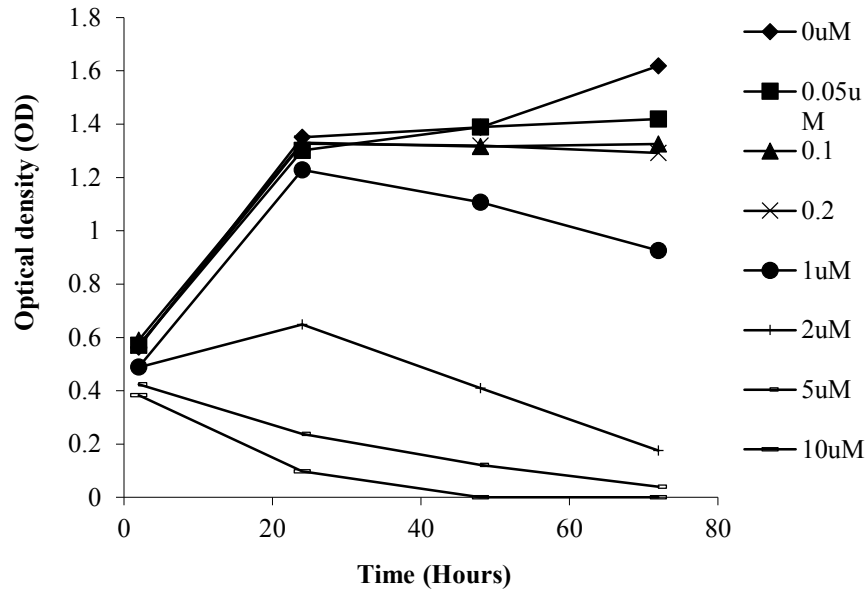
challenge site. 2 out of 6 mice developed recurrence at the original tumor site, most likely through acquired drug resistance or the activation of immune evasion pathways (Figure 3B and C). These data indicate that Sorafenib treatment induces tumor rejection that is in part dependent on T cells. Moreover, protection from a second tumor challenge suggests that Sorafenib also supports effective T cell memory responses.

Figures:

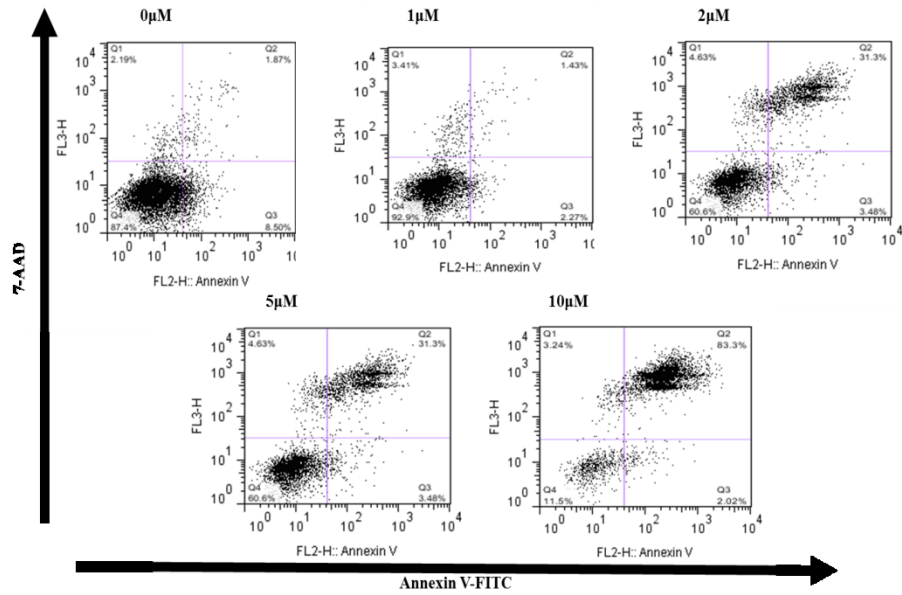
Figure 1: Sorafenib Inhibits growth of HER2-overexpressing cells *in vitro*

A, NT2.5 cells were treated *in vitro* with varying concentration of Sorafenib from 0-10 μ M and analyzed for growth by MTT assay 24, 48 or 72 hours post-treatment. **B**, NT2.5 cells were treated with Sorafenib for 24hrs and stained for Annexin V and 7-AAD and analyzed by flow cytometry. **C**, NT2.5 cells were treated with Sorafenib for 2 hours and then cells were harvested for protein and analyzed by Western blot for HER2 pathway targets. **D**, NT2.5 cells were treated with 5 μ M and 10 μ M Sorafenib or MAPK inhibitors for 6-7 hours and then cells were harvested for protein and analyzed by Western blot for cyclin expression.

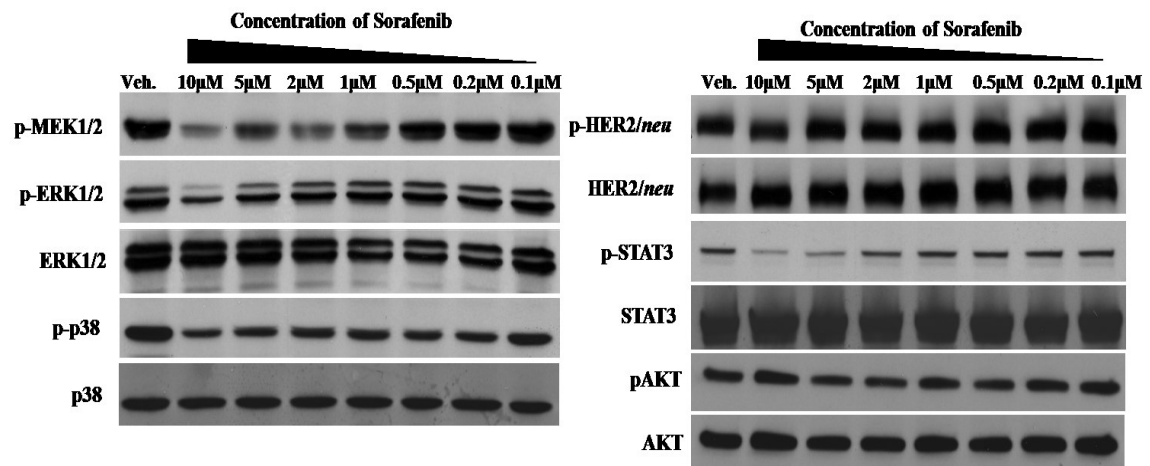
A.



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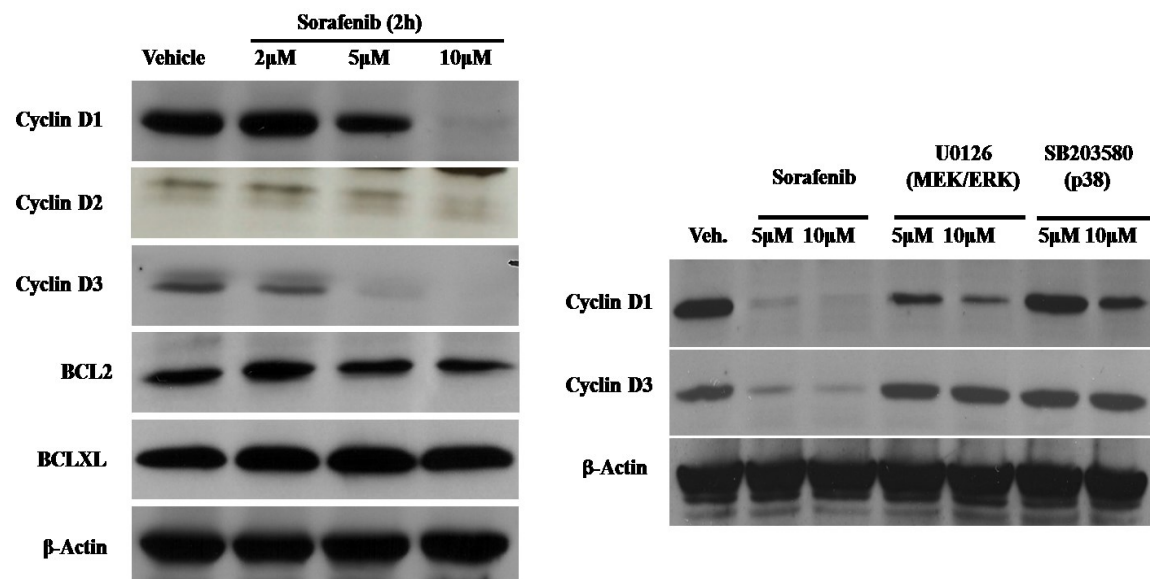
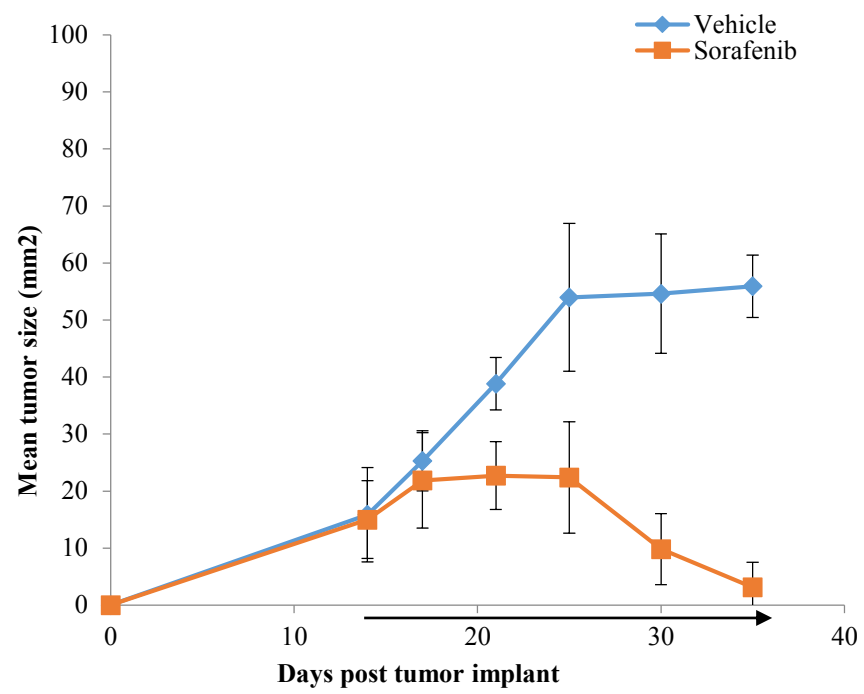


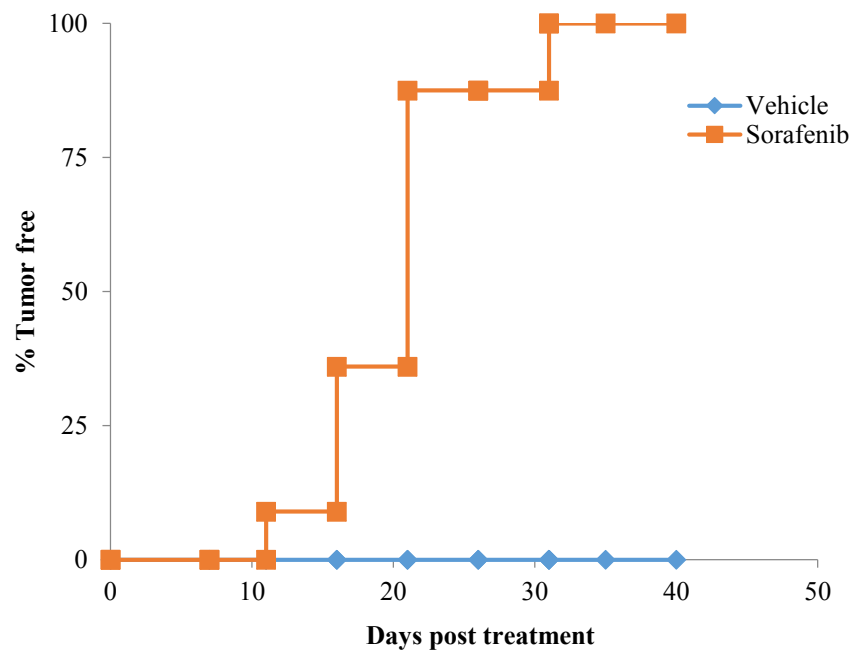
Figure 2: Sorafenib inhibits growth of breast cancer cells *in vivo*.

A and **B**, FVB/N mice (n=10) were tumor challenged at Day 0 and began Sorafenib treatment on Day 14 and followed for tumor growth and overall survival. Tumors were harvested at day 12 post-treatment and formalin fixed and paraffin embedded and stained by immunohistochemistry. Representative samples of mice treated with vehicle (top) or sorafenib (bottom) are shown with H&E staining or immunohistochemistry to detect endothelial cells (PECAM/CD31), **C**, or apoptotic cells (activated caspase 3), **D**, at 10X magnification. Staining was quantified using Elements software. Graphs (mean + SD) are cell counts from 5 samples per group, *, $P < 0.05$ and ***, $P < 0.001$.

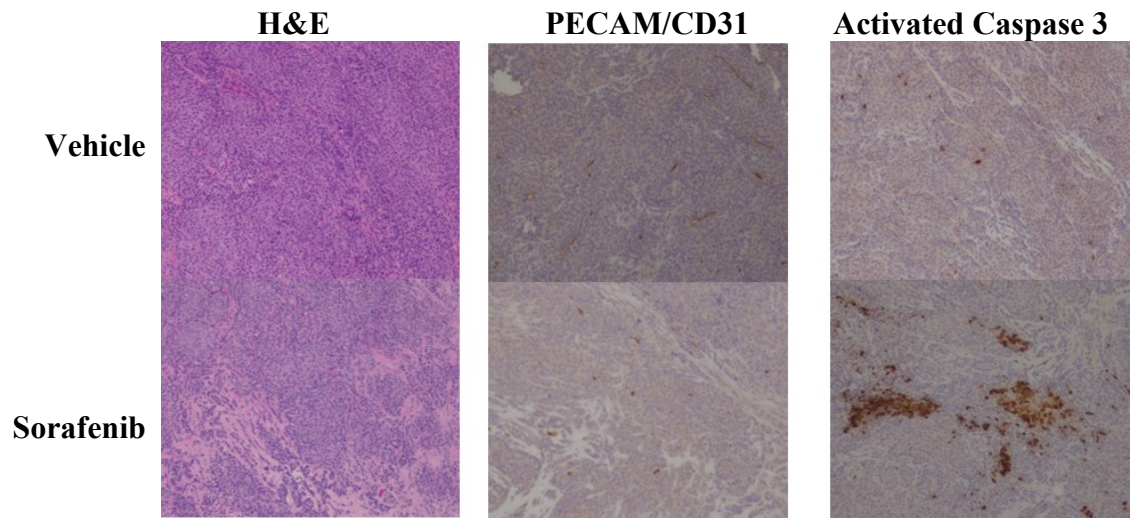
A.



B.



C.



D.

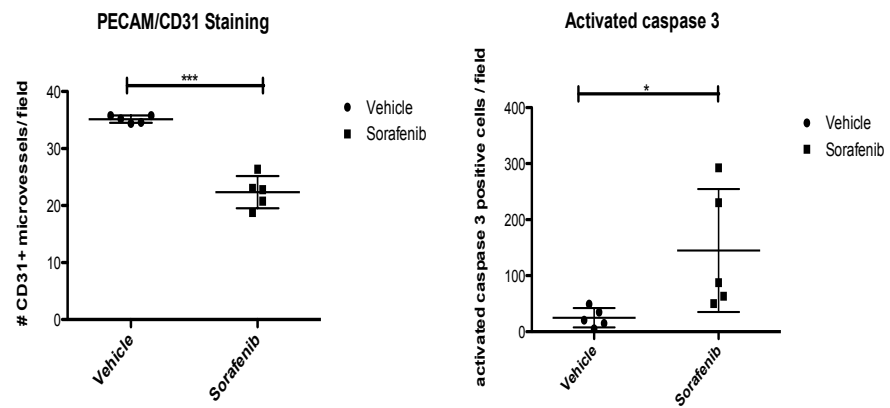
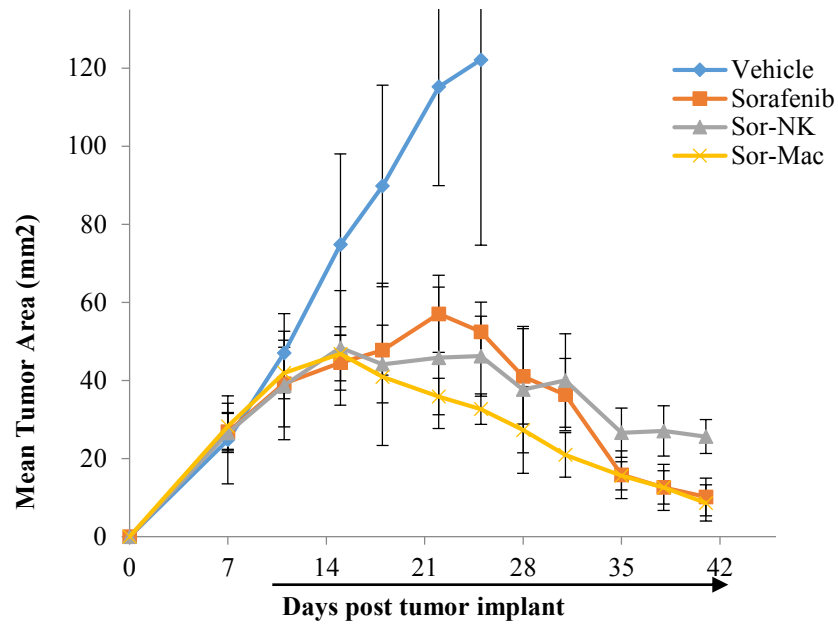


Figure 3: T cells are required for Sorafenib targeting of NT2.5 cells.

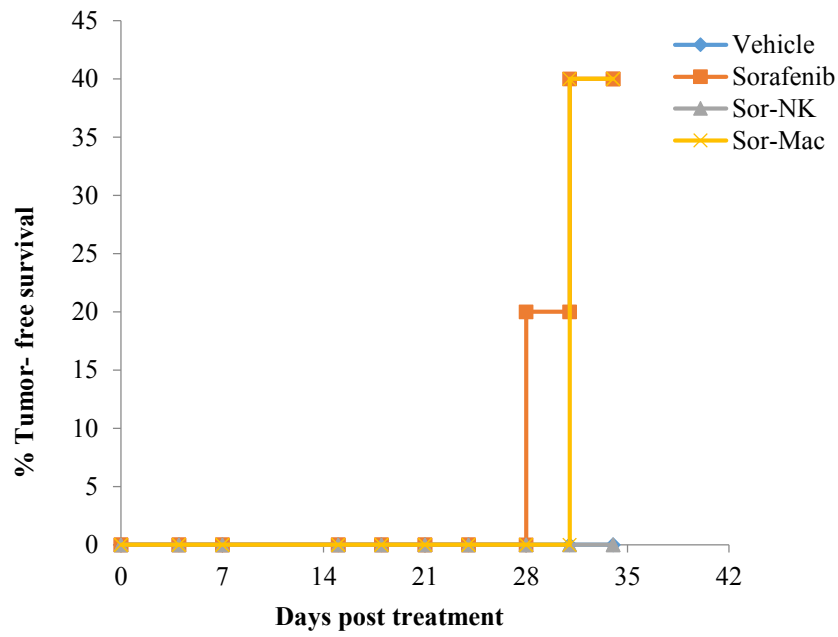
A, the experiment in Fig. 2A was repeated in the setting of immune cell depletion. Prior to beginning Sorafenib, NK cells or macrophages (Sor-NK, Sor-Mac) were depleted or **C**, CD4⁺ or CD8⁺ T cells (Sor-CD4, Sor-CD8) were depleted alone or together (Sor-CD4/CD8) and followed for tumor growth and, **B** and **D**, overall survival. **E**, in a separate experiment, FVB/N (n=6) were tumor challenged treated with Sorafenib treated or vehicle control until the Sorafenib treated tumors had completely regressed, upon which point the vehicle group was sacrificed and the Sorafenib treatment ceased. After 1 week mice were re-challenged on the contralateral side and followed for tumor growth at the original site and the re-challenge site and **F**, overall survival.

A.

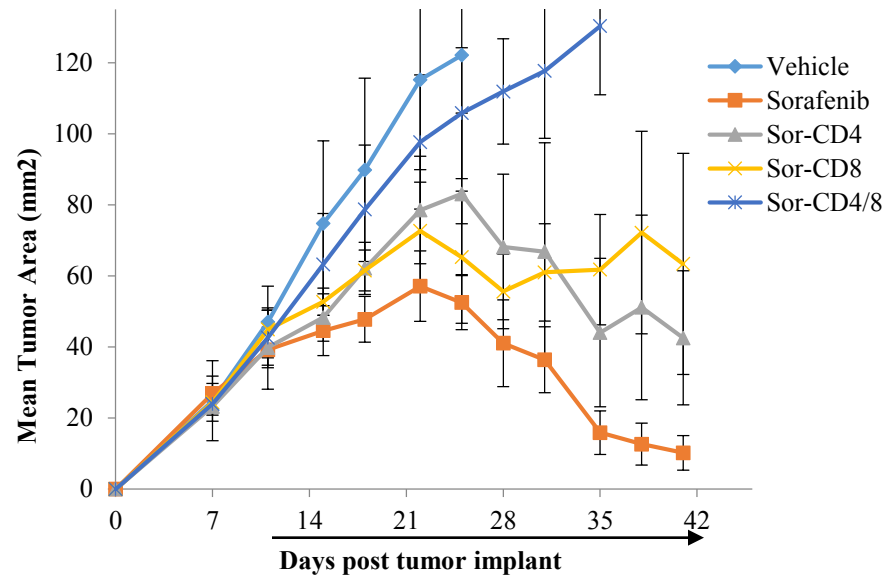


Depletion Start: Day -7
Drug Treatment Start: Day 10

B.

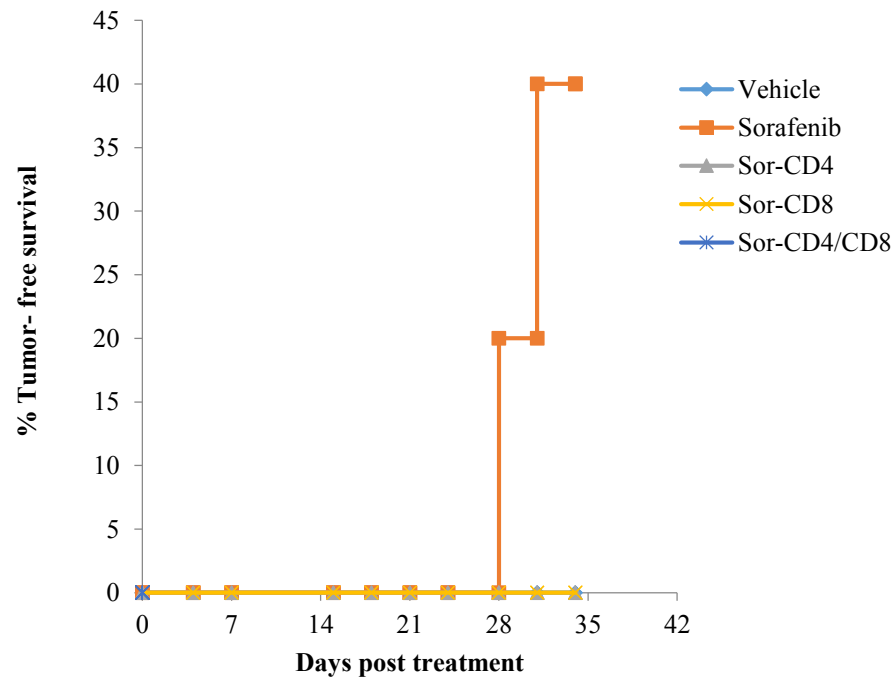


C.

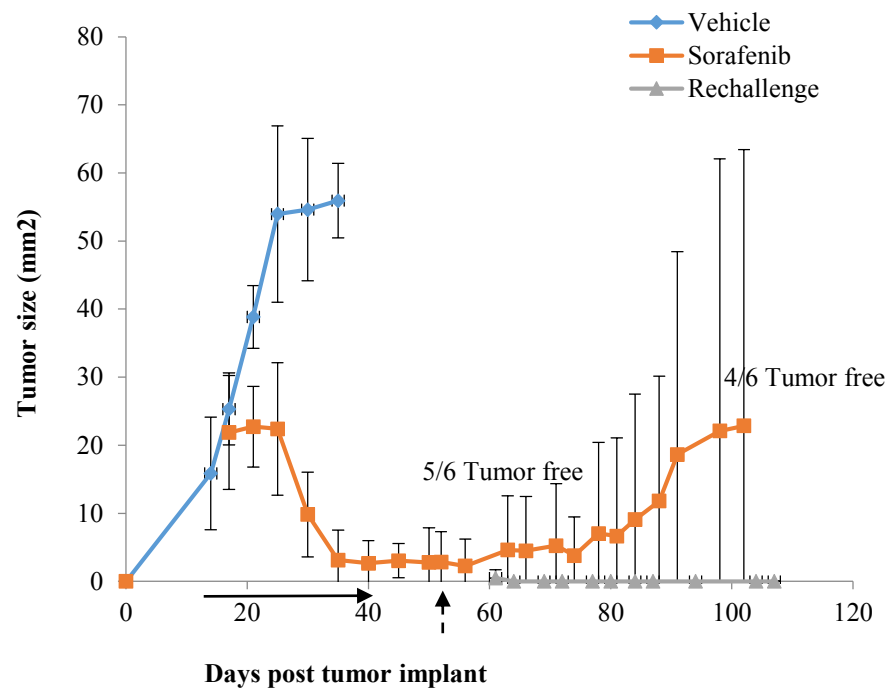


Depletion Start: Day -7
Drug Treatment Start: Day 10

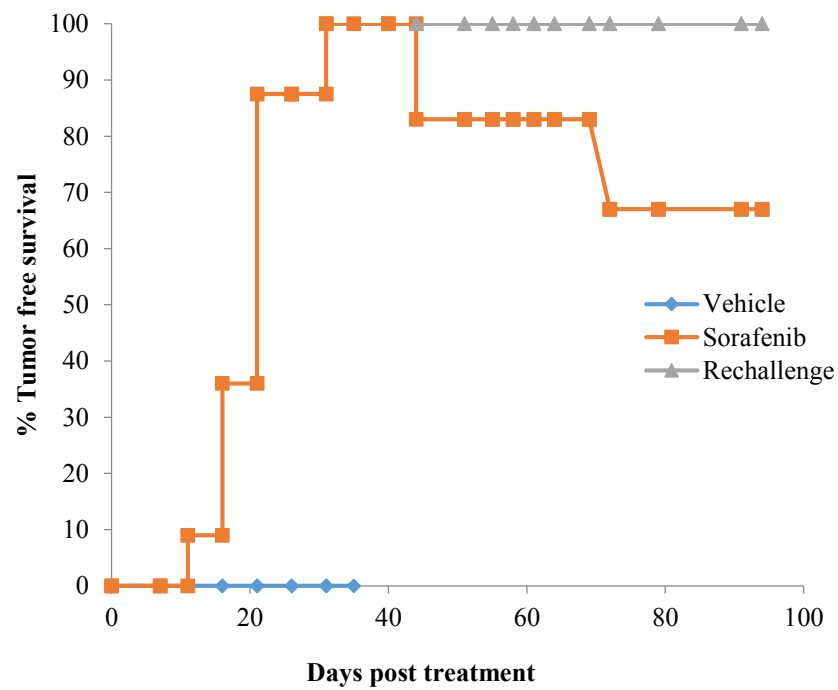
D.



E.



F.



Conclusions:

The data presented here support two new findings. First, the tyrosine kinase inhibitor Sorafenib inhibits the growth of breast cancer cells *in vitro* and *in vivo* by both MAPK dependent and independent mechanisms. Second, Sorafenib-induced tumor rejection is, in part, T cell-mediated. Both CD4⁺ and CD8⁺ T cells are required for tumor regression with single agent Sorafenib treatment. Additionally, treatment with Sorafenib supports the development of immunological memory, preventing the outgrowth of a tumor challenge. Although many studies have investigated the effect of Sorafenib on immune cells, this is the first study showing immune cell dependence for drug efficacy.

Given the multiple components of the dynamic host-tumor cell interactions within the tumor microenvironment, therapies successfully targeting multiple pathways will likely result in the most effective treatments. Here, it is demonstrated that Sorafenib inhibits the growth of HER-2-overexpressing breast tumors by a variety of mechanisms, including inhibition of cell growth, induction of cell death, and inhibition of angiogenesis. *In vitro*, clinically relevant concentrations of Sorafenib (5 μ M-10 μ M) induced marked inhibition of cell growth. Additionally, Sorafenib inhibited MAPK signaling in NT2.5 cells, likely resulting in decreased cell growth and increased cell death. These data are consistent with reported effects of Sorafenib on the Ras/MEK/ERK pathway in other cancer models^{14,24-28}. These findings were extended to show that decreased expression of the MAPK downstream target, cyclin D1, was greatest with Sorafenib treatment compared to treatment with inhibitors specific for either p38 MAPK or ERK MAPK. Unlike individual p38 MAPK or ERK MAPK inhibitors, Sorafenib treatment also decreased cyclin D3 expression. These findings suggest that Sorafenib also targets MAPK independent

pathways essential for cell cycle progression and proliferation, and is consistent with previously published reports showing that cyclin D1 and cyclin D3 are differentially regulated²³. Therapies targeting both cyclins will likely be most effective at inhibiting cell growth and successfully decreasing breast tumor burden.²⁹ In support of this, Sorafenib treatment resulted in a significant increase in cell death by increase in apoptotic cell markers *in vitro*.

In vivo, Sorafenib has been shown to inhibit tumor growth in numerous murine cancer models³⁰. Here, it is shown that daily Sorafenib treatment enhanced tumor clearance in FVB/N mice implanted with HER-2-overexpressing NT2.5 tumors. Sorafenib mediated tumor destruction through inducing cell death through apoptosis, as reflected by increased staining of activated caspase 3 in Sorafenib treated tumors. In addition to its direct tumor cell cytotoxicity, Sorafenib potently inhibits angiogenesis in NT2.5 tumors. Sorafenib treated tumors displayed substantial reduction in the number of CD31/PECAM positive microvessels, consistent with reported anti-angiogenic effects of the drug¹⁴.

In addition to its direct anti-angiogenic and cytotoxic effects, these data demonstrate that Sorafenib requires T cells to mediate durable anti-tumor activity. Simultaneously removing both CD4+ and CD8+ T cells completely abrogated the therapeutic effect of Sorafenib. Additionally, Sorafenib treatment protected mice from tumor growth after re-challenge, demonstrating immunologic memory effect. While several studies have reported on the impact of Sorafenib on the immune system^{18,20,21,31-33}, this is the first study showing a direct T cell- dependent mechanism of action for Sorafenib-mediated tumor clearance. These data build upon previously published work illustrating the influence of the immune system, specifically T cells, on the activity of anti-angiogenic therapies. Additionally, Sorafenib

can elicit long-lasting systemic immunity reflected by rejection of a second tumor challenge. Re-growth was observed in a few of the original tumors once treatment ceased. This demonstrates the dynamic immune resistance mechanisms that are active within the tumor microenvironment and suggests that it may be advantageous to combine other immune-modulating therapies with Sorafenib to enhance therapeutic benefit in patients.

Angiogenesis causes the formation of abnormal vascular networks resulting in hypoxia, increased tumor pressure, and acidosis within the tumor microenvironment. These conditions activate anti-inflammatory signals within the tumor microenvironment that support the recruitment of suppressive immune cell populations subsequently inhibiting effector cell activation. Resultant impaired APC cell maturation and migration and impaired T cell trafficking and activation dampens productive anti-tumor responses³⁴. Therefore, inhibiting angiogenesis may work to remodel the tumor microenvironment to one that is more immunosupportive rather than immunosuppressive³⁴⁻³⁶.

Given the established clinical indications for Sorafenib and the complex immune effects reported on Sorafenib, further studies are necessary to elucidate the T cell-dependent mechanism of Sorafenib. The later chapters of this thesis explore the effects of Sorafenib on two important immune cell types within the breast tumor microenvironment, T cells and TAMs. The final chapter will address these immune mechanisms in the context of using Sorafenib in combination with DC-based cellular immunotherapy.

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Chapter 3: The Immunomodulatory Effects of Sorafenib on T cells

Introduction:

Anti-tumor responses mediated by T cells are essential for successful tumor cell destruction. Antigen-targeted CTLs can exit the thymus and may be able to recognize altered self-antigens that are present on the tumor. However, peripheral immune tolerance and escape mechanisms active within the tumor microenvironment often result in impaired T cell function such as reduced cytokine production as well as hypo-responsiveness to antigenic re-stimulation^{1,2}.

In addition to overall decreased T effector cell function within the tumor, the presence of Tregs characterized by the expression of FoxP3 can also hinder productive effector T cell (Teff) responses. Tregs are a subset of CD4⁺ T cells that are specialized in suppressing T cell proliferation through the production of cytokines such as IL-10 and TGFβ³. Under normal conditions, Tregs represent up to 5% to 10% of peripheral CD4⁺ T cells and are responsible for maintaining and controlling immunological self-tolerance⁴. Increased peripheral blood CD4⁺ CD25⁺ Tregs have been reported in breast cancer patients⁵.

In addition to its cytotoxic and anti-angiogenic properties, the TKI, Sorafenib, has been reported to have T cell-modulating activity. For example, RCC patients receiving Sorafenib treatment were reported to have decreased tumor-promoting T regulatory cells in peripheral blood as well as within the tumor, resulting in increased Teff responses. Low doses of Sorafenib have been shown to increase Teff proliferation and IL-2 secretion *in vitro* and induce a Th1 dominant response *in vivo* in patients with HCC⁶⁻⁸. Conversely, studies have also shown that Sorafenib may inhibit T cell responses through inhibiting

peripheral T cell proliferation and altering LCK phosphorylation⁹. Reports also show a decrease in antigen specific T cell responses with Sorafenib treatment¹⁰. It is unknown whether or not these immune modulating effects are present during Sorafenib treatment of breast cancer.

In Chapter 2, it was demonstrated that Sorafenib treatment is effective as a single agent in enhancing tumor resolution in FVB/N mice with orthotopically implanted NT2.5 mammary tumors. Additionally, depletion studies showed the mechanism of Sorafenib is, at least in part, dependent on the presence of both CD4+ and CD8+ T cells (Ch2, Figure 3A & 3B). These studies aim to determine the effect of Sorafenib on both effector and regulatory T cells using both *in vitro* and *in vivo* murine models of HER2 over-expressing breast tumors.

It was hypothesized that Sorafenib would augment anti-tumor effector T cell responses to promote tumor clearance. To address this hypothesis, the effects of Sorafenib on CD4+ and CD8+ T cells *in vitro* were characterized. The effect of Sorafenib on Teff and Treg activation, proliferation, and cytokine production was also examined. The effect of Sorafenib on Th1 cell cytokine production specifically was examined. Finally, the effect of Sorafenib on tumor infiltrating T cells *in vivo* was analyzed. While *in vitro* studies suggest that Sorafenib inhibits T cell proliferation and cytokine secretion, *in vivo* data does not corroborate these results. Therefore, the environments in which experiments are carried out are an important factor in evaluating the effect of Sorafenib on immune cells, specifically T cells. These results reiterate the complexities of the cellular interactions within the tumor microenvironment and suggest that there may be other, yet undetermined, immune mechanisms of Sorafenib-mediated tumor clearance.

Materials and Methods:

Mice

FVB/N mice were purchased from Harlan (Frederick, MD). FVB/N FoxP3-GFP mice were maintained in house. OT-1 OVA TCR transgenic mice and 6.5 HA-TCR transgenic mice were donated from the laboratory of Dr. Charles Drake at Johns Hopkins School of Medicine. Experiments were done with 8 to 12 week old mice. Animals were housed in pathogen-free conditions and were treated in accordance with institutional and AAALAC policies. All protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

Reagents

Sorafenib was purchased from LC Laboratories (Woburn, MA). CD4 Pacific Blue, CD8 FITC, FoxP3 PE, CD25 PE, IFN γ -PeCy7, IL-17 Percp5.5, TNF α AF700, IL-2 APC, Tbet PE antibodies were obtained from eBioscience. Dynabeads Mouse T-Activator CD3/CD28 for T-Cell Expansion and Activation (Anti-CD3/anti-CD28 beads) and Carboxyfluorescein succinimidyl ester (CFSE) were purchased from Life Technologies. Human recombinant IL-2 was purchased from R&D Systems. Mouse recombinant soluble anti-mouse CD3 and CD28, and mouse recombinant IFN γ , IL-2 and IL-12 were donated by the laboratory of Dr. Jonathon Powell at Johns Hopkins School of Medicine. OVA and HA peptides were donated by the laboratory of Dr. Charles Drake at Johns Hopkins School of Medicine.

Cell Lines and Media

The NT2.5 tumor cell line, derived from a spontaneous tumor of a *neu*-N transgenic mouse, was grown as previously described¹¹.

Antigen Specific T Cell Proliferation Assays

Splenic CD8⁺ T cells from OT-1 mice or CD4⁺ T cells from HA transgenic mice were isolated by negative isolation following the package instructions (Dynabeads, Life Technologies). Cells were labeled with CFSE as per package instructions and cells were plated in 96 well at 10⁶ cells/ml with 20ng/μl IL-2 in the presence or absence of 1ng/ml of peptide (OVA, CD8⁺ T cells and HA, CD4⁺ T cells) in the presence or absence of 0.1uM, 1uM, or 10uM Sorafenib. Control wells contained equivalent amounts of DMSO. Cells were incubated at 37°C for 3 days and samples were run on Beckman Coulter Galios Flow Cytometer to analyze CFSE incorporation. 7-AAD was used to stain for live cells and analysis was performed on live cells based on 7-AAD negativity. For CD8⁺ T cell assays, half of the cells were fixed and permeabilized with FoxP3- staining buffer set (eBioscience) and stained with IFN γ APC.

Th1 skewing experiments

3μg/ml anti-CD3 in PBS was added to 6 well plates and incubated for 2 hours at 37°C. Splenic CD4⁺ T cells were negatively isolated from FVB/N mice using Dynabeads No Touch CD4⁺ T cell isolation kit (Invitrogen). 2×10^5 cells were placed in each well of a 96 well plate. IFN γ , IL-12 and IL-2 were added to induce Th1 cytokine production. All wells received 2μg/ml soluble anti-mouse CD28. Sorafenib treated wells received 8μM Sorafenib. The amount of DMSO was normalized for all wells in the experiment. Cells were incubated at 37°C for 3 days and cells were stained for CD4 and fixed and permeabilized with FoxP3 Staining Buffer Kit (eBioscience) and stained for IL-2 APC, IFN γ PECy7, TNF α AF700, and Tbet PE and run on Beckman Coulter Galios Flow Cytometer. Results were analyzed using FlowJo Analysis software.

Treg proliferation Experiments

Splenic CD4⁺ T cells from FVB/N-FoxP3-GFP were isolated by negative isolation. FoxP3⁺GFP⁺ cells (Tregs) were sorted using Cell Sorting Facility at Johns Hopkins School of Medicine. CD4⁺ FoxP3⁻ cells (Teff) were also collected. Cells were labeled with Cell Proliferation Dye eFluor® 670 (Ebioscience) and 10⁵ Tregs or Teff were plated in the presence of 4μM or 8μM Sorafenib with anti-CD3/anti-CD28 beads and 30U/ml rIL-2. The amount of DMSO was normalized for all wells in the experiment. Separately, 2×10⁵ cells were plated in triplicate for overnight culture without anti-CD3/anti-CD28 stimulation for analysis of surface marker staining of FoxP3 and CD25. After 3 days, stimulation at 37°C, the cells were stained for surface CD4 and CD4⁺ FoxP3⁺ cells were analyzed for proliferation by running samples on the Beckman Coulter Galios Flow Cytometer.

Treg Suppression Assay

Splenic CD4⁺ T cell were isolated from FVB/N mice by negative isolation. Tregs were then selected for using CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (MACs Miltenyi Biotec). Tregs were treated for 24 hours with 0mM, 0.1μM, 1μM, or 10μM Sorafenib. The following day CD4⁺ Teff were isolated from the spleens of FVB/N mice by negative isolation. Teffs were labeled with CFSE. Sorafenib was removed from Treg cultures and cells were washed with serum-free media and 10⁵ Teff cells were plated with the following ratio Teff: Treg: 1:0, 1:1, 2:1, and 5:1 in the presence 0.5μl anti-CD3/anti-CD28 beads. This amount was experimentally determined to be appropriate to allow suppression of proliferation in the presence of Treg cells at a 1:1 ratio of Tregs to Teffs. Teffs were analyzed for CFSE after 3 days in culture by flow cytometry.

Tumor-infiltrating T cell analysis

FVB/N mice were challenged subcutaneously with 5×10^6 NT2.5 tumor cells in the right mammary fat pad, followed by treatment 10-14 days later. Sorafenib (30mg/kg) was administered in 100 μ l daily Monday through Friday by oral gavage with a feeding needle. A viscous vehicle composed of 30% (w/v) Cremophor EL, 30% (w/v) PEG 400, and 10% ethanol, 10% glucose (Sigma-Aldrich) was used both to dissolve Sorafenib and administered as the vehicle treatment control. Mice were monitored for tumor growth and onset twice weekly. Tumor growth was determined by measuring tumor diameter in two perpendicular dimensions with calipers. Mice were treated for 12 days and then tumors and spleens were harvested. At this time point, there was a statistically significant difference in tumor size between vehicle and Sorafenib treated tumors (Chapter 2, Figure 3A & 3B). Tumors were digested with Liberase TM (Roche) for 30 minutes and the single cell suspension was stained for CD4 and CD8 and then fixed and permeabilized and stained for intracellular IL2, IFN γ , FoxP3, IL17, and TNF α (eBioscience) and run on the Beckman Coulter Galios Flow Cytometer. Results were analyzed with FlowJo analysis software.

Statistical Analysis

Statistical analysis was conducted either in Microsoft Excel or GraphPad Software using an unpaired, two-tailed Student's t-test, assuming equal population variances to determine the statistical significance between treatment groups. $P < 0.05$ was considered to be significant

Results:

Sorafenib inhibits antigen specific T cell proliferation *in vitro*

In order to determine potential immunomodulatory effect of Sorafenib on T cells, the effect of Sorafenib on T cell proliferation, cytokine production, and function *in vitro* was examined. OT-1 splenic CD8⁺ T cells pulsed with OVA peptide were used to analyze antigen-specific CD8⁺ T cells as it is a well-established model of antigen-specific CD8⁺ T cell proliferation and it has been previously used to study the effect of Sorafenib on T cell proliferation *in vitro*^{10,12}. There was a concentration dependent decrease in the amount of OVA-specific CD8⁺ T cell proliferation with increasing concentrations of Sorafenib (Figure 4A). Sorafenib treatment also resulted in a decrease in proliferation of CD8⁺ T cells in culture in the absence of peptide. IFN γ production by CD8⁺ T pulsed with OVA peptide also decreased with Sorafenib treatment (Figure 4B).

To analyze antigen-specific CD4⁺ T cell responses, HA-specific T cell responses were measured in 6.5 splenic CD4⁺ T cells. This model is a well-established model of antigen-specific CD4⁺ T cell proliferation *in vitro*¹³. High concentrations of Sorafenib resulted in decreased proliferation in CD4⁺ T cells pulsed with HA peptide (Figure 4C). Taken together, these data confirm previous studies that Sorafenib can impair T cell proliferation *in vitro*^{6,9,10}.

Sorafenib inhibits cytokine production of Th1 CD4⁺ T cells *in vitro*

Productive anti-tumor immune responses usually coincide with a Th1-type immune response, indicated by the presence of Th1- CD4⁺ T helper cells that secrete pro-inflammatory cytokines such as IFN γ , TNF α , IL-2, and lymphotoxin¹⁴. Therefore, the

ability of Sorafenib to promote the production of Th1 cytokines, TNF α , IL-2, and IFN γ in CD4 $^{+}$ T cells was examined *in vitro*. CD4 $^{+}$ T cells in the presence of Th1 cytokines and soluble α CD28 showed increased IFN γ when compared to control cells that received soluble α CD28 alone. Treatment of splenic CD4 $^{+}$ T cells with Th1 skewing cytokines and soluble α CD28 in the presence of Sorafenib resulted in decreased production of TNF α , IL-2, and IFN γ . Specifically, IFN γ production decreased from 35% to 15% with Sorafenib treatment. CD4 $^{+}$ T cells that received only α CD28 alone displayed increased TNF α and IL-2 production when compared to cells treated with Th1 skewing cytokines in the presence of Sorafenib. (Figure 5A-5C). The effect of Sorafenib treatment on Tbet, the master-regulator transcription factor of Th1 cell fates, was also analyzed (Figure 5D). Treatment with Sorafenib did not alter Tbet expression in the CD4 $^{+}$ T cells in the presence of Th1 cytokines and soluble α CD28.

Sorafenib alters the proliferation, activation, and function of Tregs *in vitro*

Reduction in number or function of suppressive Treg populations can result in enhanced T cell-mediated tumor clearance. Preferentially targeting Tregs, leaving effector cell populations unharmed, have been shown to be an effective mechanism to improve anti-tumor immunity in Sorafenib- treated HCC patients⁸. Therefore, the ability of Sorafenib to target CD4 $^{+}$ Treg proliferation was explored *in vitro*. Sorafenib treatment of splenic CD4 $^{+}$ FoxP3 $^{+}$ Tregs resulted in decreased cell proliferation in response to TCR stimulus with α CD3/ α CD28 beads. A decrease in proliferation was similarly observed in CD4 $^{+}$ FoxP3 $^{-}$ T effector cells treated with Sorafenib (Figure 6A). Taken together, these data

indicate that *in vitro* treatment of CD4⁺ T cells with Sorafenib results in diminished cell proliferation, irrespective of cell function.

Phenotypic markers CD25 and FoxP3 have been shown to correlate with the suppressive capacity of Tregs^{15,16}. Therefore, the effect of Sorafenib treatment on the expression of CD25 and FoxP3 in Tregs was also analyzed. The percentage of CD25⁺ FoxP3⁺ Tregs was largely unchanged after 24 hours treatment with Sorafenib (Figure 6B & 6C). However, increasing concentrations of Sorafenib did cause a significant decrease in relative expression of both CD25 and FoxP3 measured by decreased mean fluorescence intensity by flow cytometry analysis (Figure 6D & 6E).

To further examine if the observed decrease in these Treg phenotypic markers corresponded to altered Treg suppressive activity in this system, Sorafenib-treated Tregs were analyzed for suppressive function *in vitro*. Tregs were treated with Sorafenib 24 hours prior to the addition of CFSE-labeled CD4⁺ effector cells (Teff) at increasing cell ratios of Tregs to effector cells. At lower Treg to Teff cell ratios (1:5), higher concentrations of Sorafenib significantly decreased the suppressive capacity of Tregs in culture compared to untreated Tregs as evidenced by increased CD4⁺ effector T cell proliferation at these ratios (Figure 6F). Taken together, these data indicate that Sorafenib decreased the proliferation and activation of Tregs *in vitro* as well as decreased their suppressive capacity at physiological Treg to Teff cell ratios. However, Sorafenib is not potent enough to inhibit Tregs at high Treg to Teff cell ratios (1:1).

Sorafenib does not affect infiltration or cytokine production of TILs

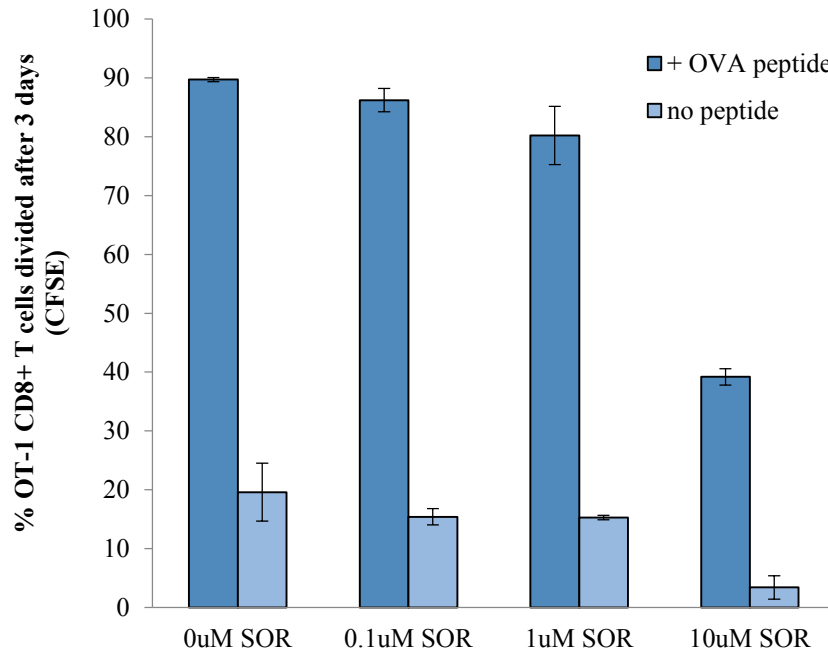
The effect of Sorafenib on tumor-infiltrating T cells (TILs) was then examined. TILs from NT2.5 tumors in FVB/N mice treated with Sorafenib or vehicle control were analyzed by flow cytometry. TILs isolated from tumors of Sorafenib-treated mice and vehicle treated mice showed a relatively similar composition of both CD8⁺ T cells and CD4⁺ T cells. Intracellular staining showed no differences in the cytokine profiles of CD8⁺ or CD4⁺ T cells. Finally, there was no difference in the percentage of CD4⁺FoxP3⁺ T cells infiltrating the tumors of either treatment group.

Figures:

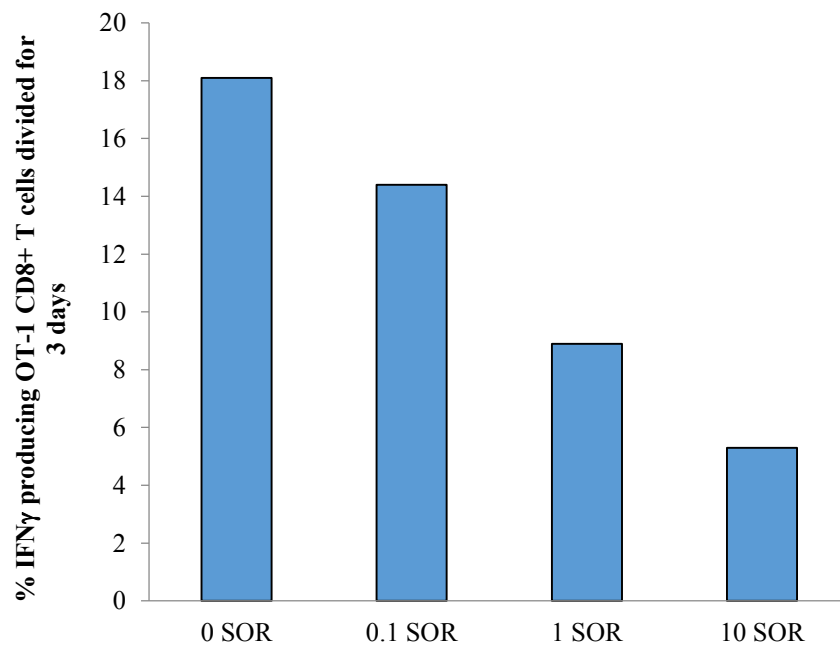
Figure 4: Sorafenib inhibits antigen specific T cell proliferation and cytokine production *in vitro*.

A, Proliferation of CFSE-labeled splenic CD8⁺ from OT-1 transgenic mice in response to OVA peptide stimulation for 3 days in the presence or absence of Sorafenib. **B**, IFN γ production of the CD8⁺ T cells from **A**. **C**, Proliferation of CD4⁺ T cells from 6.5 transgenic mice in response to HA peptide stimulation for 3 days in the presence of Sorafenib.

A.



B.



C.

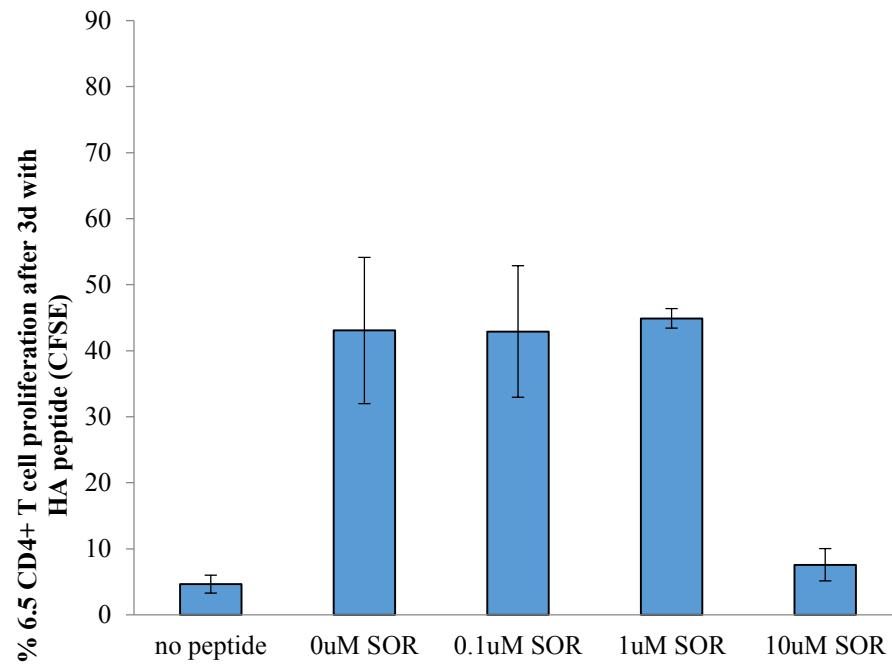
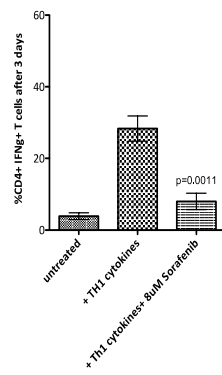


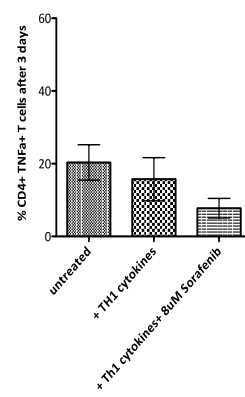
Figure 5: Sorafenib inhibits cytokine production of Th1-skewed cells *in vitro*

FVB/N splenic CD4⁺ T cells were negatively isolated and plated at with or without Th1-skewing cytokines IFN γ , IL-12 and IL-2 in the presence of 2 μ g/ml soluble anti-mouse CD28 and plate-bound anti-mouse CD3. Sorafenib treated wells received 8 μ M Sorafenib. After 3 days cells were analyzed by ICS for IFN γ **A**, TNF α , **B**, and IL-2, **C**. Tbet levels, **D**, and relative expression was also analyzed, **E**.

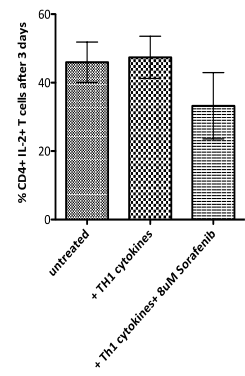
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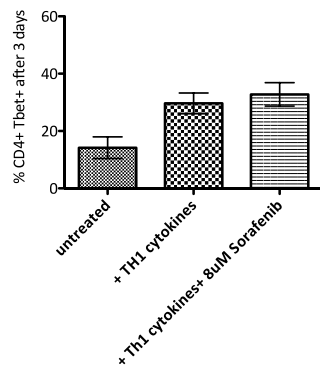
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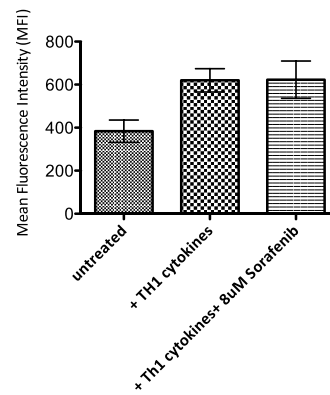
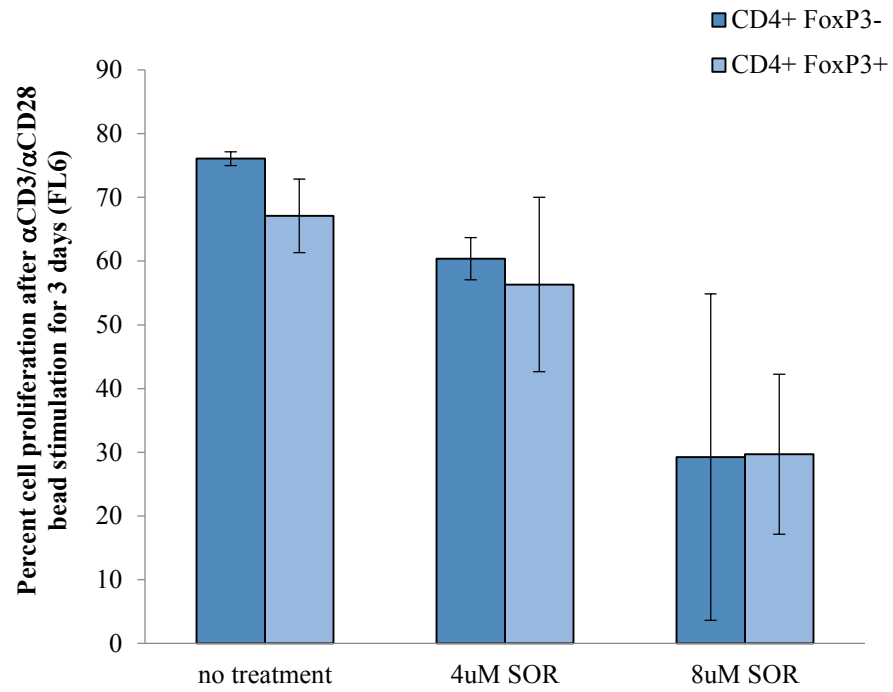


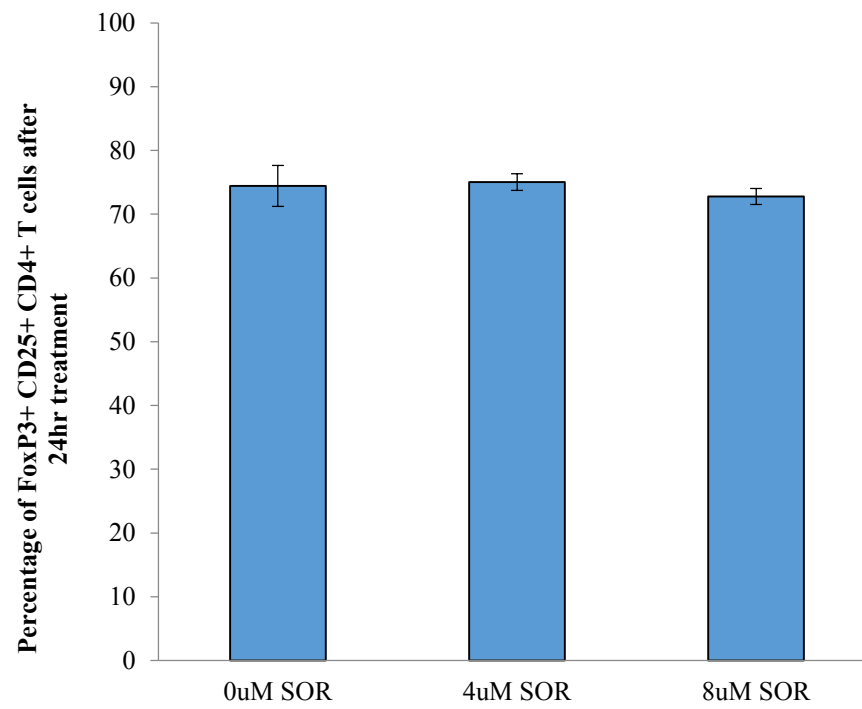
Figure 6: Sorafenib alters the proliferation, activation, and function of Tregs *in vitro*

A, Splenic FoxP3+GFP+ CD4+ T cells (Tregs) and CD4+ FoxP3- CD4+ T cells (Teff) were collected. 10^5 Tregs or Teff were plated in the presence of 4 μ M or 8 μ M Sorafenib with 0.5 μ l anti-CD3/anti-CD28 beads and proliferation was analyzed after 3 days in culture. **B**, 2×10^5 FoxP3+GFP+ CD4+ cells were plated in triplicate for overnight culture with Sorafenib and stained for surface markers FoxP3 and CD25. Relative expression of FoxP3, **C**, and CD25, **D**, was also analyzed. CD4+ CD25+ cells were used as Tregs and treated overnight in culture with 0 μ M, 0.1 μ M, 1 μ M, and 10 μ M Sorafenib. After 24 hours, CD4+ Teff were isolated and were labeled with CFSE. Sorafenib was removed from Treg cultures and Teff cells were plated at the following ratios of Treg:Teff, with 10^5 Teff staying constant: 0:1, 1:1, 1:2, and 1:5 in the presence of 0.5 μ l of anti-CD3/anti-CD28 beads. **E**, Proliferation was analyzed after 3 days by CFSE incorporation in Teffs. *, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.001$.

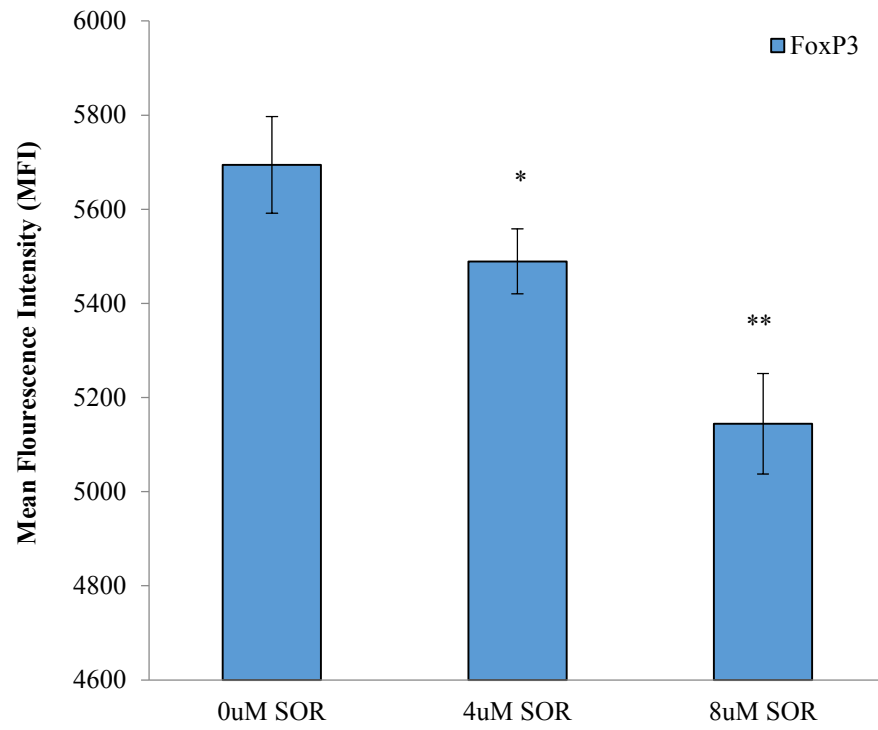
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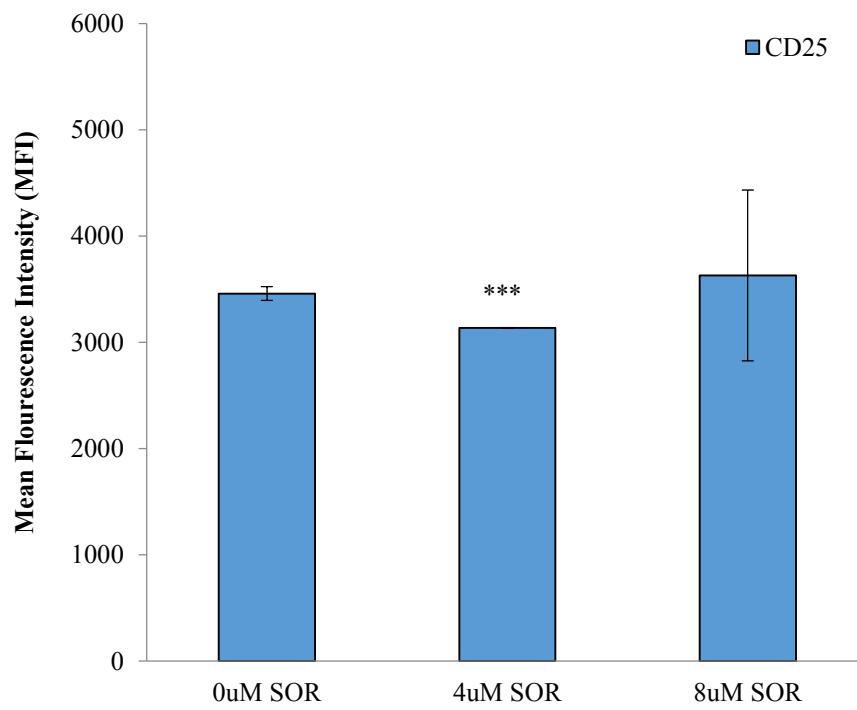
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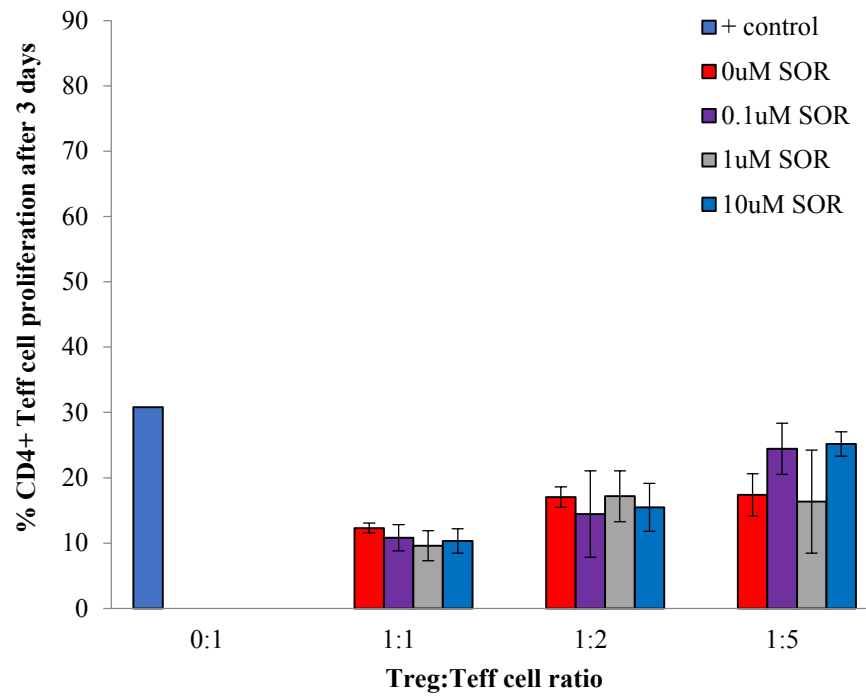
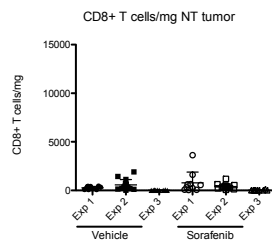
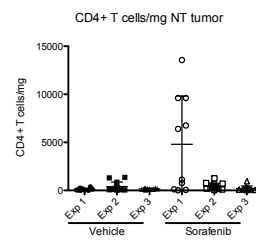
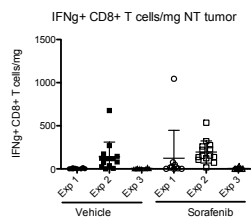
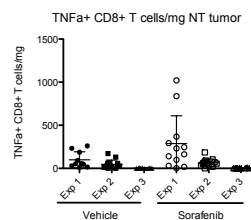
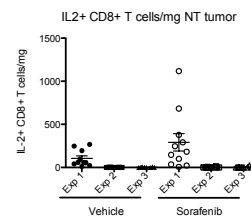
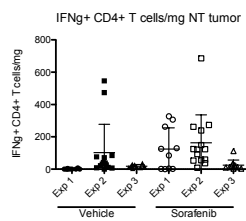
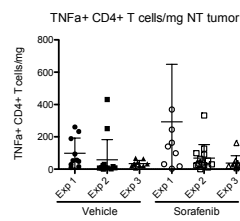
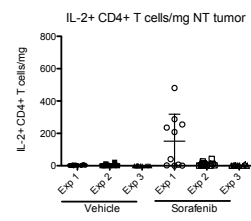
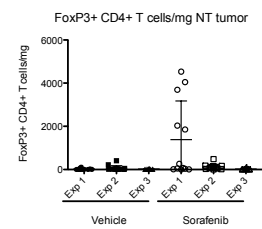


Figure 7: Sorafenib does not alter tumor-infiltrating T cell number or cytokine production *in vivo*

FVB/N mice were challenged subcutaneously with 5×10^6 NT2.5 tumor cells. After 10 days, mice were treated with Sorafenib (30mg/kg) for 12 days and then tumors were harvested and digested. Single cell suspension was stained for **A**, CD4 and **B**, CD8 and then fixed and permeabilized and stained for intracellular , IFN γ , **C** and **F**, TNF α , **D** and **G**, IL2, **E** and **H**, and FoxP3, **I**.

A.**B.****C.****D.****E.****F.****G.****H.****I.**

Conclusions:

The data presented here confirm previous findings that Sorafenib inhibits T cell proliferation and cytokine production *in vitro*. Additionally, these data expand upon previous studies, elucidating a potential mechanism by which Sorafenib may inhibit Tregs *in vitro* through alterations in activation markers, CD25 and FoxP3, decreasing suppressor function. Lastly, these data support a new finding that, in contrast to *in vitro* observations, Sorafenib treatment does not inhibit T cell infiltration or cytokine production at the tumor site *in vivo*.

The ability of Sorafenib to inhibit T cells has been well documented. In 2008, Hipp et al. showed inhibition of OVA-specific CD8⁺ T cell responses *in vivo* in mice pre-treated with Sorafenib followed by peptide vaccination with adjuvant¹⁰. In the same year, W. Zhao and colleagues published that off target effects of Sorafenib target LCK phosphorylation, thereby inhibiting the activation of human peripheral T cells⁹. In 2009, R. Houben and colleagues showed a decrease in survivin-specific T cell responses in melanoma patients vaccinated against survivin with Sorafenib treatment⁶. In Chapter 2, it was reported that Sorafenib treatment augmented NT2.5 tumor clearance in FVB/N mice by a T cell-dependent mechanism. Given the myriad proposed T cell effects potentiated by Sorafenib, it was necessary to evaluate the *in vitro* and *in vivo* effects of Sorafenib in this model system.

The previously published OT-1-OVA system and 6.5-HA system were used to analyze *in vitro* effects of Sorafenib on T cell proliferation. Both antigen specific CD8⁺ and CD4⁺ T cells showed decrease in proliferation in response to peptide with Sorafenib treatment.

CD8⁺ IFN γ production also decreased in the presence of Sorafenib. These data are consistent with prior studies demonstrating that Sorafenib decreased T cell proliferation and cytokine production *in vitro*^{6,9,10}. The data presented here also expands upon these studies to analyze the effect of Sorafenib specifically on Th1 cytokine production. While Sorafenib treatment decreased the production of Th1 cytokines, specifically, IFN γ it does not affect the expression of Tbet. This suggests that Sorafenib may not be permanently affecting licensing of CD4⁺ T cells to become Th1 cells, however this remains unknown.

Conversely, several groups have shown a positive impact of Sorafenib treatment through modulating T regulatory cells both in mice and humans^{7,8,17-19}. In HCC patients receiving Sorafenib therapy, decreased peripheral Tregs corresponded to increased clinical benefit¹⁸. The *in vitro* data presented here support these previous findings showing that Sorafenib can decrease Treg proliferation, phenotypic activation markers, and function. *In vitro*, Sorafenib inhibits proliferation and significantly decreases the expression of CD25 and FoxP3 on Tregs. This decrease in expression of activation marker corresponds to a decrease in suppressive function. At physiologically relevant cell ratios of Treg to T_{eff}, Sorafenib treatment results in a significant decrease in the ability of Tregs to suppressive the proliferation of CD4⁺ T effector cells. It is also possible that at lower ratios of Tregs:T_{eff}, Sorafenib pre-treatment of Tregs altered cell viability, therefore, these results may also be explained by a diminished number of Tregs rather than decreased suppressive capacity. This could be reconciled in the future by verifying the viability of the Tregs at the assay endpoint.

In contrast to *in vitro* observations, *in vivo*, there were no differences between the absolute numbers of infiltrating T cells, nor were there measurable differences in the cytokine profiles of CD4+ or CD8+ T cells as a result of Sorafenib treatment. Sorafenib treatment does not inhibit T cell alter trafficking of T cells to the tumor, nor does it modify cytokine production by T cells at the tumor site. Additionally, there were no differences in the number of tumor-infiltrating FoxP3+ T cells, contrasting reports published in HCC^{18,19}. It remains unknown as to whether Sorafenib treatment affects the function of tumor-infiltrating Tregs *in vivo*.

Mounting a successful immune response within the tumor microenvironment is complex, the mechanisms of which are still under active investigation. Immune-mediated tumor clearance relies on the ability of effector T cells to enter the tumor, secrete cytokines and actively kill tumor cells. According to the data presented here, Sorafenib does not improve T cell infiltration into the tumor nor does it enhance cytokine production by effector cells. Additionally, it does not reduce the percentage of suppressive Treg at the tumor site. However, the effect of Sorafenib on other effector functions of the infiltrating cells besides cytokine secretion, such as killing, remains unknown. Additionally, it is possible that Sorafenib may be targeting other cells within the tumor to enhance clearance by T cells. For example, Sorafenib-mediated tumor cell death may elicit increased danger signals to promote enhanced tumor clearance^{20,21}. Additionally, Sorafenib may be altering co-stimulatory molecules to augment recognition of the tumor²². Also, Sorafenib treatment may induce changes within antigen presenting cells in the tumor microenvironment, allowing for improved priming and increased antigen presentation within the tumor. To

this end, the next chapter explores the immunomodulatory effects of Sorafenib on tumor-associated macrophages.

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Chapter 4: The Immunomodulatory Effects of Sorafenib on Tumor-associated Macrophages

Introduction:

Macrophages are mononuclear phagocytes of the innate immune system that defend the host against harmful pathogens and heal tissues after injury. Macrophages also regulate tissue growth, homeostasis and repair through the expression and release of a variety of growth factors and cytokines. Macrophages phagocytose microbes and present antigens to T cells, orchestrating the acute inflammatory response to eliminate the invading pathogens¹. Additionally, macrophages play a role as scavengers that clear tissue debris. Given their diverse function, macrophages play a central role in inflammation, tissue remodeling, cell growth and angiogenesis; many of these roles are known to promote tumor progression^{2,3}.

Macrophages are grouped into subsets based on the acquisition of distinct morphological and functional properties directed by particular tissues or immunological microenvironment^{4,5}. Classically activated inflammatory macrophages (M1) are induced by IFN γ alone or combined with microbial stimuli, such as lipopolysaccharide (LPS), or with other cytokines such as TNF and GM-CSF. These cells have an IL-12^{high}, IL-23^{high}, IL-10_{low} phenotype⁶. Moreover, these cells are also efficient producers of effector molecules such as reactive oxygen and nitrogen intermediates and inflammatory cytokines such as IL-1 β , TNF and IL-6. Consistent with these functional characteristics, M1 macrophages participate in Th1 responses and help mediate resistance to intracellular infections and tumors⁷.

In contrast, M2 or alternatively activated tissue tropic macrophages differentiate in microenvironments rich in Th2 cytokines such as IL-4 and IL-13 and in tissues to promote growth and development⁸. These cells generally have high levels of scavenger, mannose, and galactose-type receptors⁴. Arginase expression is also increased as result in a shift in arginine metabolism to produce ornithine and polyamines. In general, M2 cells participate in Th2 reactions, that promote killing and encapsulation of parasites, tissue repair, and remodeling⁹⁻¹¹.

Lastly, a third subset of regulatory macrophages has been described. Regulatory macrophages exhibit an IL-12^{low}, IL-23^{low}, IL-10^{high} phenotype. This results in the secretion of high levels of anti-inflammatory interleukin IL-10 and low levels of pro-inflammatory IL-12/23. Prostaglandin E₂ (PGE₂), extracellular adenosine, immune complexes, VEGF, IL-10, and TGF- β , can all drive the regulatory macrophage phenotype¹². It has been shown that mitogen-activated protein kinase ERK plays a key role in this process¹³⁻¹⁵. Under conditions of strong ERK activation, the anti-inflammatory cytokine IL-10 is upregulated and pro-inflammatory IL-12/23 is suppressed¹⁶. Because IL-10 can inhibit the production and activity of various pro-inflammatory cytokines, these regulatory macrophages are potent inhibitors of inflammation.

Macrophages are a major cellular component of breast tumors, where they have been reported to compose as much as fifty percent of the infiltrating cells. In the tumor, they are commonly termed tumor-associated macrophages (TAMs). These macrophages change their physiology and take on a phenotype that more closely resembles regulatory macrophages¹⁷. The tumor-derived agents that induce the development of these regulatory macrophages have not been identified, but candidates include prostaglandins, hypoxia,

extracellular nucleotides, apoptotic cells, hyaluronan fragments and IgG¹⁸⁻²⁰, which may work synergistically within the tumor microenvironment. Irrespective of the stimulus, these tumor-associated macrophages produce high levels of IL-10, can inhibit immune responses to neo-antigens expressed by tumor cells, and can de-activate neighboring macrophages²¹. Recent studies also suggest that regulatory macrophages can contribute to angiogenesis and thereby promote tumor growth¹⁸. Clinical and experimental evidence has shown that cancer tissues with high infiltration of TAM are associated with poor patient prognosis and resistance to therapies²². There is also evidence that macrophage depletion in some cases may even be beneficial to the host²³. Given the role of TAMs in tumor progression, targeting of macrophages in tumors is considered a promising therapeutic strategy, whereby depletion of TAMs or their ‘re-education’ as anti-tumor effectors is under current investigation.

Accumulating data suggest that, in addition to inhibiting tumor cell proliferation and angiogenesis, Sorafenib can modulate immune cell function, specifically macrophage function. It has been previously shown that Sorafenib treatment can shift bone-marrow derived macrophages activated with LPS and PGE₂ from the pro-tumorigenic IL-10 secreting phenotype to the anti-tumor IL-12 secreting phenotype²⁴. Additionally it was found that Sorafenib treatment enhances proinflammatory activity of TAMs and subsequently induces antitumor NK cell responses in a cytokine and NF-κB-dependent fashion.²⁵ Conversely, in a murine HCC model Sorafenib treatment significantly increased peripheral recruitment and intratumoral infiltration of F4/80+CD11b+ cells and elevated pro-tumoral and pro-angiogenic factors in the tumor and peripheral blood, suggesting a role of macrophages in tumor progression under Sorafenib treatment.

Depletion of macrophages in combination with Sorafenib treatment significantly inhibited tumor progression, tumor angiogenesis, and metastasis compared with mice treated with Sorafenib alone. Here, these studies are extended to explore the effect of Sorafenib on TAMs in a murine model of breast cancer.

First, the impact of Sorafenib on TAM recruitment within the tumor was analyzed. Then, the effect of Sorafenib treatment on TAM cytokine production was examined. Finally, functional analysis on Sorafenib-treated TAMs was performed. These data suggest that Sorafenib may alter the activation state of TAMs to increase pro-immunogenic cytokines and enhance CD4⁺ T cell proliferation.

Materials and Methods:

Mice

FVB/N mice were purchased from Harlan (Frederick, MD). Experiments were done with 8 to 12 week old mice. Animals were housed in pathogen-free conditions and were treated in accordance with institutional and AAALAC policies. All protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

Reagents

Sorafenib was purchased from LC Laboratories (Woburn, MA). CD11b FITC, MHCII PE, CD4 Pacific Blue, and CD8 Pacific Blue antibodies were obtained from eBioscience. Carboxyfluorescein succinimidyl ester (CFSE) were purchased from Life Technologies. Mouse recombinant soluble anti-mouse CD3 and CD28 were donated by the laboratory of Dr. Jonathon Powell at Johns Hopkins School of Medicine.

Cell Lines and Media

The NT2.5 tumor cell line, derived from a spontaneous tumor of a *neu*-N transgenic mouse, was grown as previously described²⁶.

Drug treatment

FVB/N mice were challenged subcutaneously with 5×10^6 NT2.5 tumor cells in the right mammary fat pad, followed by vaccination 10-14 days later. Sorafenib (30mg/kg) was administered in 100 μ l daily Monday through Friday by oral gavage with a feeding needle beginning the day of vaccination. A viscous vehicle composed of 30% (w/v) Cremophor EL, 30% (w/v) PEG 400, and 10% ethanol, 10% glucose (Sigma-Aldrich) was used both to dissolve Sorafenib and administered as the vehicle treatment control.

Immunohistochemistry

Tumors harvested at day 12 post-treatment were fixed in formalin for 24 hours, paraffin-embedded, and sectioned at 5µM by the JHMI Pathology Core. Sections were stained with H&E or retained for immunohistochemistry at the JHMI Oncology Tissue Service Center. F480 was analyzed with antibodies specific for F480 (Cell Signaling). Antigen retrieval was carried out for 45 minutes in HTTR steam (Target Retrieval Solution; Dako) followed by incubation with primary antibody for 45 minutes at room temperature. Slides were incubated with Power Vision Poly-HRP anti-rabbit IgG secondary antibody for 30 minutes at room temperature. Slides were developed with 3, 3' diaminobenzidine (Sigma Fast DAB tablets) and slides were counterstained with Mayers hematoxylin (Dako). Images were captured under light microscopy at 20x magnification (E600, Nikon).

Tumor-associated macrophage preparations

Tumors were harvested day 12 post-treatment and suspended in 5 ml RPMI 1640 containing Liberase TM (Roche). Tumors were minced and incubated at 37°C for 30 min. Suspensions were then passed through a 100-µm mesh nylon cell strainer (BD Falcon) to obtain single cell suspensions. Single cell suspensions were incubated with ACK lysing buffer (Sigma) for 2 min at room temperature and washed twice in FACS-staining buffer (PBS, 2% heat-inactivated FCS). Pellets were resuspended in FACS staining buffer containing Fc Block (MACs Miltenyi Biotec) and incubated on at 4°C for 20 minutes. TAMs were incubated with FACS buffer containing biotinylated anti-F480 (eBioscience) and isolated by positive selection (MACs Miltenyi Biotec).

Cytospins

10^4 TAMs were resuspended in 1ml PBS. Cells were spun onto chambered microscope slides at maximum speed for 5 minutes. Slides were allowed to dry and stained with Diff-Quick, following the manufacturers instructions, to visualize morphology.

Quantitative real-time PCR

mRNA was extracted from T cells with TRIzol Reagent (Life Technologies) following the manufacturer's protocol. cDNA was synthesized with a RNA to cDNA with EcoDry Premix kit (Clontech). All primers were purchased from Life Technologies-Applied Biosystems; reactions were performed in triplicate using an Applied Biosystems StepOnePlus Instrument.

TAM Suppression Assay

Splenic CD4⁺ T cells or CD8⁺ T cells were isolated from FVB/N mice by negative isolation. Tregs were then selected from the CD4⁺ T cell population using CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (MACs Miltenyi Biotec). Tumors were harvested from FVB/N mice on day 12 post-treatment. Tumors were minced and digested in Liberase TM (Roche) for 45 minutes. Cells were filtered through 100 μ M filters to obtain a single cell suspension. Red blood cells were lysed using ACK lysis buffer (Sigma). TAMs were isolated by positive selection for F480 using biotinylated anti- (MACs Miltenyi Biotec). T cells were labeled with CFSE. 10^5 T cells were plated with the following ratio T cell: TAM: 1:0, 1:1, 2:1, and 5:1 in the presence 1 μ g/ml of anti-CD3 2 μ g/ml anti-CD28. T cells were also plated with at a 1:1 ratio with Tregs as a suppression control for the assay. T cells were analyzed for CFSE after 4 days in culture by flow cytometry.

Statistical Analysis

Statistical analysis was conducted either in Microsoft Excel or GraphPad Software using an unpaired, two-tailed Student's t-test, assuming equal population variances to determine the statistical significance between treatment groups. $P < 0.05$ was considered significant.

Results:

Sorafenib treated tumors seemed to show an increase in F480+ tumor-infiltrating macrophages with increased activation morphology

First, the purity of TAMs isolated from tumor tissue had to be verified (Figure 8A-C). TAMs were obtained through F480+ isolation. FACS analysis post-isolation showed a population of cells that were ninety percent CD11b+ and fifty percent positive for MHC class II. Morphology of TAMs was confirmed by cytopspin (Figure 8C).

The effect of Sorafenib treatment on TAM infiltration was analyzed by immunohistochemistry. TAMs were isolated from the NT2.5 tumors that were implanted into FVB/N mice on day 12 post-treatment with Sorafenib or vehicle control. This timepoint has been shown previously to result in tumor sizes that are significantly different between Sorafenib and vehicle treated mice (Chapter 2, Figure 2). Tumors of Sorafenib treated mice showed an increase in F480+ cells by immunohistochemistry analysis (Figure 9A). Additionally, TAMs from Sorafenib treated tumors showed an increased in activated morphology upon analysis of cytopspin compared to TAMs isolated from vehicle treated mice (Figure 9B).

Sorafenib enhances M1 cytokine secretion in tumor-associated macrophages

The effect of Sorafenib on the expression of macrophage polarity genes was then analyzed by qrt-PCR. Qrt-PCR analysis of TAMs isolated from Sorafenib treated tumors showed an increase in expression of two M1 genes, *IL-12* and *IL-6*, when compared to TAMs isolated from vehicle treated tumors (Figure 10A-D). Sorafenib treatment also resulted in a modest increase in *arg1* expression in TAMs (Figure 10E). *IL-10* expression, a marker

of regulatory macrophages, was decreased in TAMs isolated from Sorafenib treated tumors compared with the vehicle control (Figure 10F).

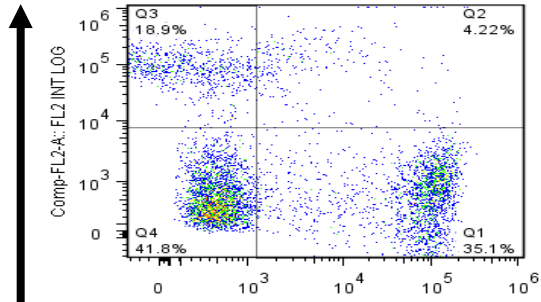
Sorafenib treated macrophages increase CD4+ T cell activation

To determine if the observed changes in cytokine gene expression corresponded to altered function, the effect of Sorafenib treatment on TAM suppressive function was then analyzed. Increasing ratio of TAMs from Sorafenib-treated tumors resulted in increased CD4+ T cell proliferation in response to α CD3/ α CD28 stimulus compared to vehicle control TAMs and CD4+ T cells cultured in the absence of TAMs (Figure 11A). TAMs from vehicle treated tumors suppressed CD4+ T cell proliferation at all cell ratios. However, TAMs do not seem to suppress CD8+ T cell proliferation (Figure 11B). The presence of TAMs from either vehicle-treated or Sorafenib-treated tumors resulted in enhanced CD8+ T cell proliferation in response to α CD3/ α CD28 stimulus.

Figures:

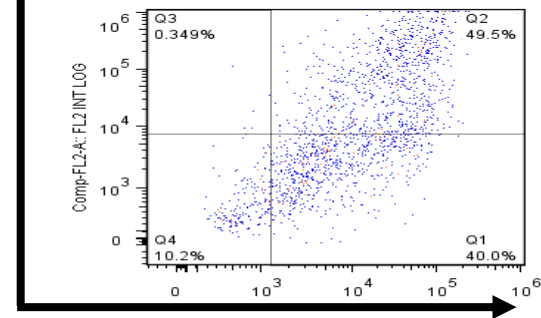
Figure 8: **Schema for Macrophages isolation from FVB/N tumors.** **A**, Splenocytes were isolated from an FVB/N tumor bearing mouse and analyzed by flow cytometry for CD11b and MHC class II as a staining control for macrophages isolated from tumors. **B**, F480+ isolated TAMs were stained for Cd11b and MHC class II. **C**, Cytospin of F480+ cells was performed to confirm macrophage morphology.

A.



B.

MHC-II PE



CD11b-FITC

C.

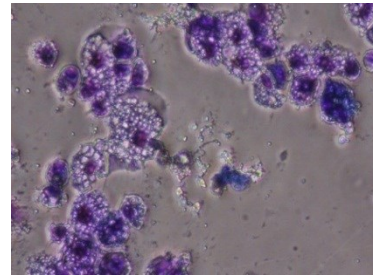
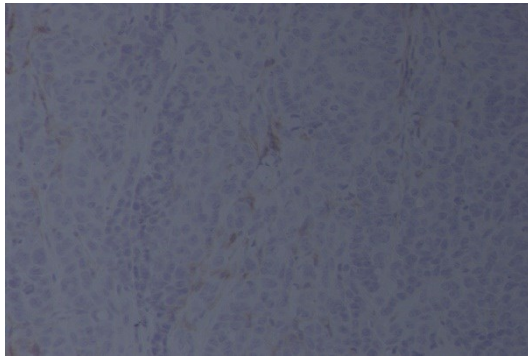


Figure 9: Sorafenib treatment increased F480+ cells in the tumor and alters TAM morphology. Tumors were harvested at day 12 post-treatment and formalin fixed and paraffin embedded and stained by immunohistochemistry for F480. Representative samples of mice treated with vehicle, **A**, or Sorafenib, **B**, are shown at 20X magnification. F480+ cells were isolated from tumors at day 12-post treatment and cytopins were performed on 10^4 F480+ TAMs from the tumors of vehicle, **C**, or Sorafenib, **D**, treated mice.

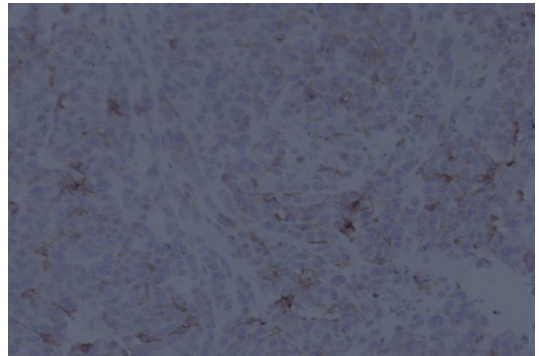
Vehicle

A.

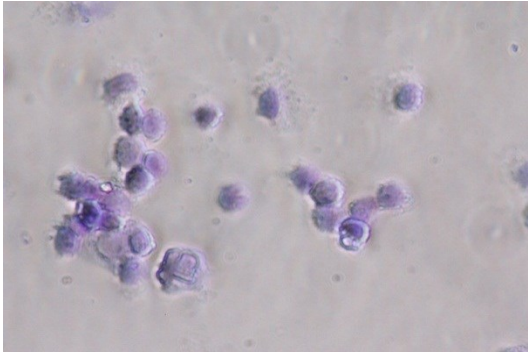


Sorafenib

B.



C.



D.

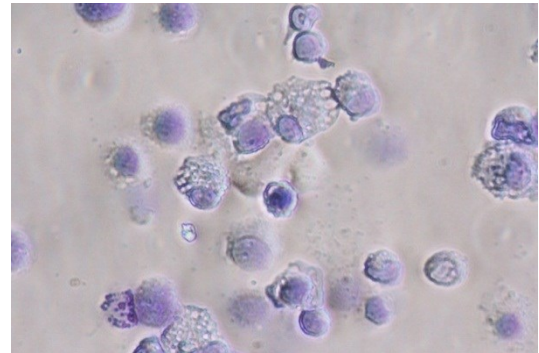
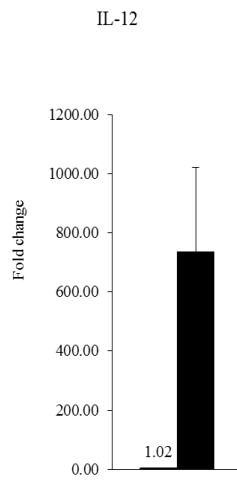


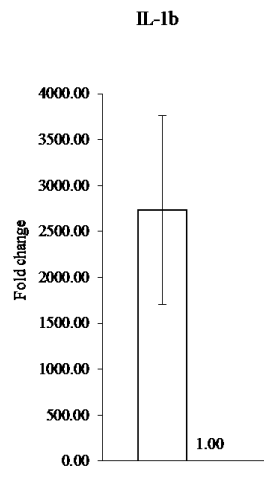
Figure 10: Sorafenib treatment enhances M1 cytokine expression in TAMs. Cytokine gene expression to analyze macrophage polarity in F480+ TAMs isolated from tumors of vehicle (white bars) or Sorafenib (black bars) treated mice was performed by qrt-PCR. M1 markers IL-12, **A**, IL-1 β , **B**, iNOS, **C**, and IL-6, **D**, were by analyzed. M2 marker, arg1, **E**, was analyzed. Regulatory marker, IL-10, **F**, was analyzed.

A.

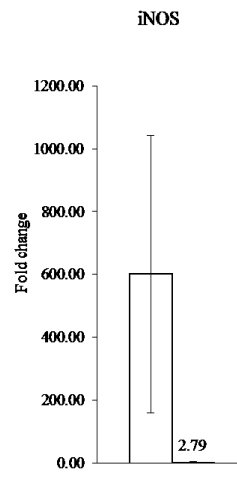


B.

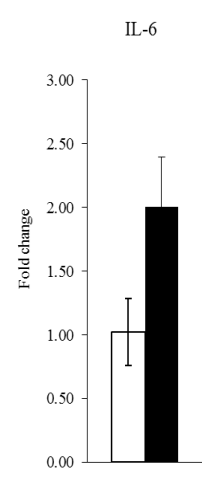
M1 Markers



C.



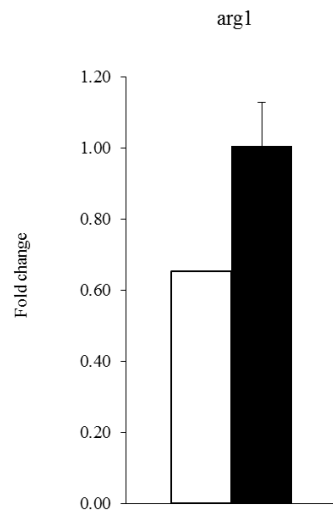
D.



□ Vehicle ■ Sorafenib

E.

M2 Marker



F.

Regulatory Marker

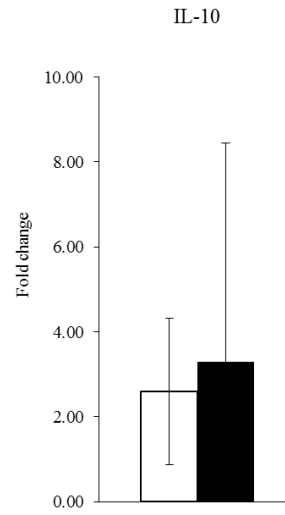
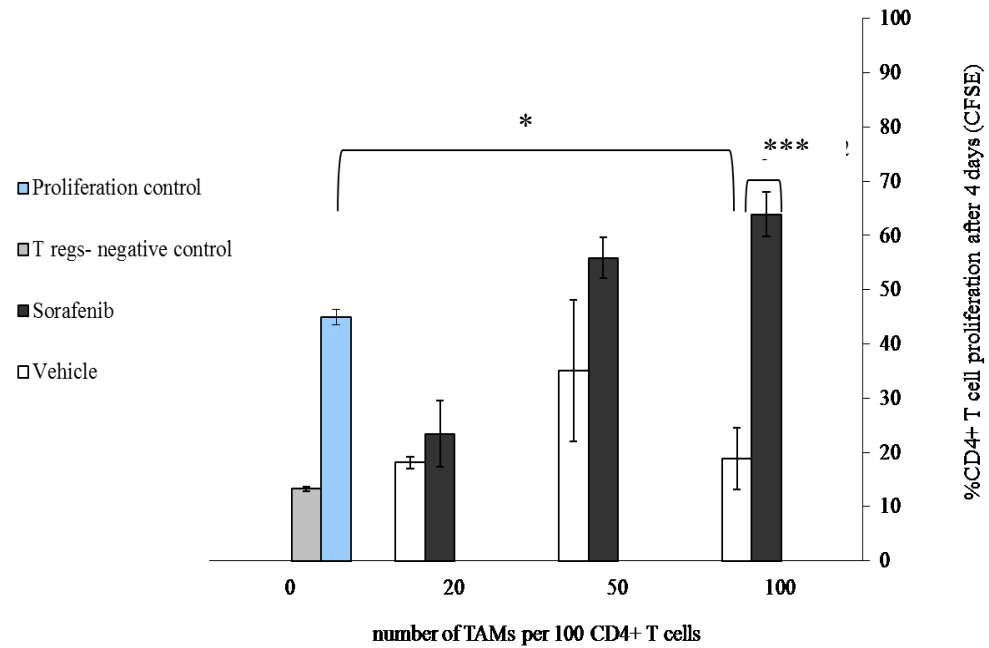


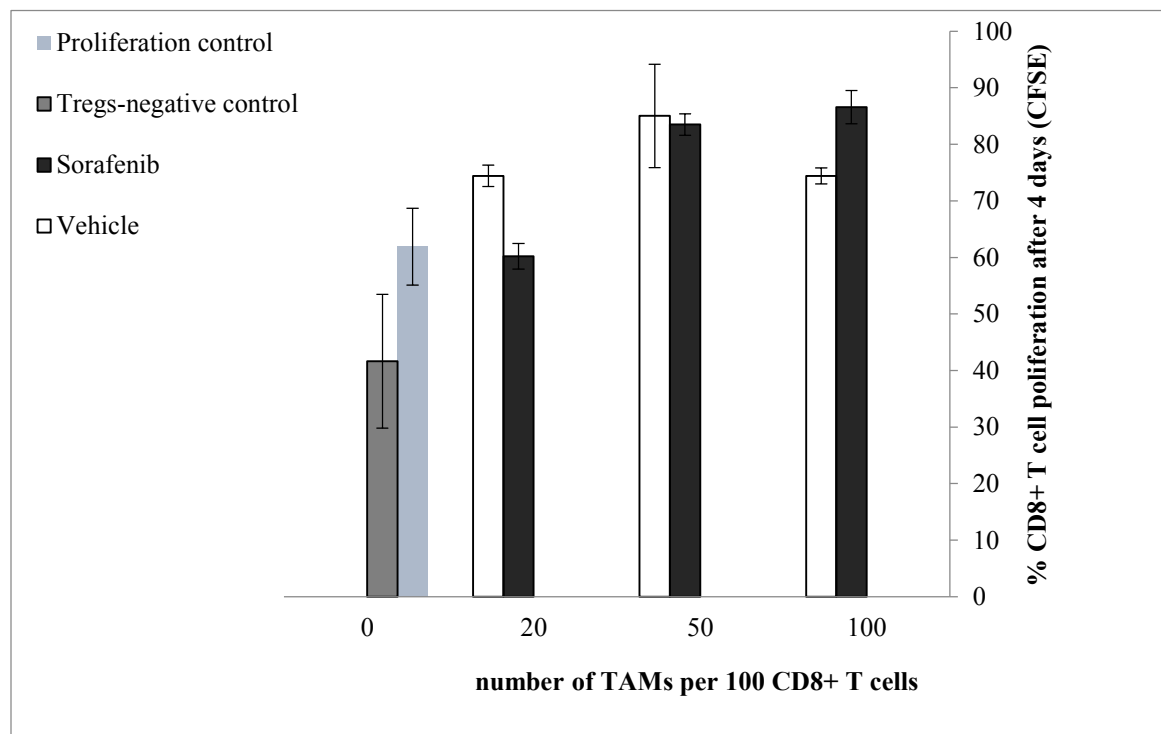
Figure 11: TAMs from Sorafenib treated tumors enhance CD4+ T cell proliferation.

A, F480+ TAMs were isolated from tumors of vehicle (white bar) or Sorafenib (black bar) treated mice. Splenic CD4+ T cells were isolated and labeled with CFSE. 10^5 CD4+ T cells were plated in the presence of TAMs at the following cell ratios, with 10^5 T cells staying constant: 0:1 (blue bar-Proliferation control), 1:1, 1:2, and 1:5 in the presence of 0.5 μ l of anti-CD3/anti-CD28 beads. Proliferation was analyzed after 3 days by CFSE incorporation in CD4+ T cells. Tregs (gray bar) were also isolated and plated with CD4+ T cells as a suppression control for the assay, *, $P < 0.05$, ***, $P < 0.001$. **B**, the experiment in **A**, was repeated with CD8+ T cells as effector cells.

A.



B.



Conclusions:

The present study supports three new findings. First, the tyrosine kinase inhibitor, Sorafenib, induces increased infiltration of F480+ TAMs into NT2.5 tumors implanted into FVB/N mice. Second, Sorafenib is able to alter cytokine expression of TAMs to increase IL-12 and decrease IL-10. Lastly, Sorafenib treated TAMs may enhance the proliferation of CD4+ T cells.

Studies have reported on the role of TAMs in tumor progression, therefore, therapies that can target TAMs may prove to have a therapeutic benefit. Sorafenib has been reported to increase the recruitment of TAMs into the peripheral blood of HCC patients. The data shown here agrees with the previous reports, showing an increase infiltration of F480+ TAMs in the tumor of FVB/N mice treated with Sorafenib.

However, in contrast to previous data, prt-PCR of inflammatory cytokine shows that while TAMs from vehicle treated mice have a regulatory phenotype (IL-10 hi IL-12 low), Sorafenib treatment alters this gene expression to resemble classically activated macrophages (IL-12 high and IL-10 low). Therefore, macrophages that enter the tumor in Sorafenib treated mice may be playing a beneficial role in supporting tumor clearance through enhanced secretion of pro-inflammatory cytokines. Edwards, et al, has previously published on the ability of Sorafenib to skew bone marrow derived macrophages *in vitro*²⁴. These data support these findings and extend them to include macrophages within the tumor.

Additionally, functional analysis of TAMs shows in the absence of Sorafenib, vehicle treated TAMs suppress CD4+ T cell proliferation, supporting the suppressive nature of

regulatory macrophages within the microenvironment of breast tumors. Treatment with Sorafenib enhanced the ability of TAMs to stimulate CD4⁺ T cell proliferation. TAMs isolated from the tumors of both treatment groups equally enhanced CD8⁺ T cell proliferation. This may be explained by the strength of α CD3/ α CD28 stimulus on inducing CD8⁺ T cell proliferation. Alternatively, this data may indicate that antigen presentation on MHC class I molecules remains unhindered on TAMs to promote CD8⁺ T cell proliferation. However, inhibitory interactions between macrophages and CD4⁺ T cells within the tumor microenvironment may prevent necessary T cell help to sustain CD8⁺ T cell proliferation. The induction of pro-inflammatory cytokines or other co-stimulatory molecules may promote CD4⁺ T cells to provide enhanced CD8⁺ T cell help to enhance tumor clearance. This would suggest that Sorafenib may have an effect on MHC class II expression, but that remains unknown. While the mechanism of Sorafenib does not rely solely on macrophages (Chapter 2), this data suggests that Sorafenib may be re-educating TAMs to support more potent anti-tumor responses.

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Chapter 5: Sorafenib Can Be Effective Combined with Cellular Immunotherapy

Introduction:

Advances in the treatment for metastatic breast cancer have improved the quality of life and conferred a small survival benefit for some patients. However, disease relapse often occurs due to development of drug resistance and, ultimately, metastatic disease remains incurable¹. Therefore, there is a need for new therapeutic strategies to evade the development of drug resistance in these patients. Consequently, ongoing efforts have focused on recruiting patients' own immune cells as a therapeutic partner to combat disease.

Cancer vaccines aim to reprogram host immune cells to become more efficient at targeting and killing cancer cells, leaving normal cell unharmed. A whole cell granulocyte macrophage colony-stimulating factor (GM-CSF)-secreting vaccine targeting HER-2 was designed to enhance the immune response to breast cancer². However, central and peripheral tolerance mechanisms limit the efficacy of vaccination. Multiple studies have shown an increase in vaccine activity by strategically combining vaccine with other cancer therapeutics to take advantage of both the cytoreductive potential of cancer drugs and the ability to interrupt immunoregulatory networks and support productive anti-tumor immune responses³⁻⁵.

Accordingly, a vaccination strategy incorporating multi-kinase inhibitors that target both the tumor cells, and other distinct cellular components within the tumor microenvironment is being developed. A novel immune-modulating activity for the Sorafenib has been

previously reported (Chapter 2). Sorafenib alone cures tumor-bearing FVB/N mice through both anti-angiogenic and immune effects.

These studies have been extended to explore the activity the small molecule multi-kinase inhibitor, Sorafenib. This study aims to determine the efficacy of combining Sorafenib with whole cell GMSCF-secreting breast cancer vaccine in a pre-clinical model. Anti-tumor immunity and tumor regression were characterized following Sorafenib treatment in combination with vaccine. Additionally, immune cell infiltrate was analyzed in single agent and combination therapy tumors. Sorafenib in combination with vaccine enhanced tumor clearance and promoted increased overall survival relative to single agent therapy. Additionally, Sorafenib treatment did not inhibit productive vaccine-induced immune responses. Finally, Sorafenib treatment enhanced vaccine-induced tumor clearance by increasing the accumulation of antigen-specific T cells at the tumor site.

Materials and Methods:

Mice

FVB/N mice were purchased from Harlan (Frederick, MD). Clone 100 T-cell receptor (TCR) transgenic mice, derived from FVB/N mice, express the high-avidity, RNEU₄₂₀₋₄₂₉–specific TCR in the majority of peripheral CD8⁺ T cells, and were generated as previously described⁶. Eight to twelve week old mice were used in the experiments. Animals were housed in pathogen-free conditions and were treated in accordance with institutional and AAALAC policies. All protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

Reagents

Sorafenib was purchased from LC Laboratories (Woburn, MA). FoxP3 PE-Cy5, CD25 PE, CD4 FITC, CD8 PE, GR1 PE, CD11b FITC, and Thy1.2 APC antibodies were obtained from eBioscience.

Cell Lines and Media

The HER-2-expressing NT2.5 breast tumor cell line (derived from a spontaneous tumor explanted from a *neu*-N transgenic mouse), the GM-CSF–secreting vaccine cell lines, 3T3GM (mock) and 3T3neuGM (HER-2-specific), and the T2D^q line were grown as previously described². The cell lines used as T-cell targets, 3T3neuB7.1 and NT2.5B7.1, were produced via retroviral transduction as previously described⁷.

Immunohistochemical staining

Tumors were fixed in formalin for 24 hours and paraffin embedded and sectioned at 5µM at the JHMI Pathology Core. Sections were stained with H&E or retained for immunohistochemistry at the JHMI Oncology Tissue Service Center. Cellular infiltrate staining was performed using antibodies for CD3, FoxP3, Gr1, and F480 (Cell Signaling). Immunohistochemistry was done with the Power Vision+ poly-HRP IHC Kit (ImmunoVision Inc). Antigen retrieval was carried out for 45 minutes in HTTR steam (Target Retrieval Solution; Dako) followed by incubation of primary antibody for 45 minutes at room temperature. Slides were incubated with Power Vision Poly-HRP anti-rabbit IgG secondary antibody for 30 minutes at room temperature. Slides were developed with 3, 3'-diaminobenzidine (Sigma Fast DAB tablets) and slides were counterstained with Dako Mayer's hematoxylin (Sigma). Images were captured under light microscopy at 20X magnification (E600, Nikon). Three independent viewing fields were captured and staining was quantified using AR-Elements Microscope Imaging Software (Nikon).

Drug treatment, vaccinations, and chemotherapy

FVB/N mice were challenged subcutaneously with 5×10^6 NT2.5 tumor cells in the right mammary fat pad, followed by treatment 10-14 days later. Sorafenib (30 mg/kg) was administered daily Monday through Friday by oral gavage with a feeding needle. A viscous vehicle composed of 30% (w/v) Cremophor EL, 30% (w/v) PEG 400, and 10% ethanol, 10% glucose (Sigma-Aldrich) was used as the Sorafenib diluent, and also administered as the vehicle treatment control. 3×10^6 vaccine cells per mouse were irradiated before subcutaneous injection in both hind limbs and the left front limb. Doses and timing for

tumor cells and vaccinations have been previously optimized². For combination treatment studies, Sorafenib treatment began on the day of vaccination. Mice were monitored for tumor growth and onset twice weekly. Tumor growth was determined by measuring tumor diameter in two perpendicular dimensions with calipers. Mean tumor size for an experimental group included only those mice with measureable tumors.

ELISPOTS

ELISPOTS were performed 14-17 days post-vaccination. Splenic CD8⁺ T cells were isolated from individual mice (not pooled) using the Dynabeads Untouched Mouse CD8 Cell Negative Isolation Kit (Life Technologies). RNEU₄₂₀₋₄₂₉ (PDSLRDLSVF) and NP₁₁₈₋₁₂₆ (RPQASGVYM) peptides were synthesized at 95% purity by the Oncology Peptide Synthesis Facility (Johns Hopkins, Baltimore, MD). 10⁵ CD8⁺ T cells were incubated in triplicate with 10⁴ peptide loaded T2D^q, NT2.5B7.1, or 3T3neuB7.1 target cells. NT2.5B7.1 and 3T3neuB7.1 cells were stimulated with IFN γ for 2 days prior to co-culture. T cell/T2D^q cells were co-cultured for 16 hours and T cell/NT2.5B7.1 or T cell/3T3neuB7.1 cells were co-cultured for 24 hours on pre-coated IFN- γ ELISPOT Multiscreen-HA plates (Millipore) according to the manufacturer's protocols (Ebioscience). ELISPOT plates were developed using an AEC staining kit (Sigma) according to the manufacturers' instructions. IFN γ -secreting CD8⁺ T cells were enumerated using the Immunospot counter (Cellular Technology, Ltd.). The average number of spots in control wells was subtracted from the average number of spots in each well containing both CD8⁺ T cells and targets.

Adoptive Transfer Experiment

Adoptive transfer was carried out as described previously⁸. Briefly, FVB/N mice received subcutaneous injections of 5×10^6 NT2.5 cells into the right upper mammary fat pad. One week post tumor challenge, mice were vaccinated with 3×10^6 irradiated 3T3neuGM cells or 3T3GM cells. Sorafenib (30 mg/kg) or vehicle was given by daily gavage starting on the day of vaccination. CD8⁺ T cells were isolated from Clone 100 TCR transgenic mice by CD8 negative selection using Dynabeads Untouched Mouse CD8 negative isolation kit (Life Technologies). 4×10^6 CD8⁺ T cells per mouse were adoptively transferred via tail vein injection one day following initiation of treatment. Spleens, lymph nodes, and tumors from adoptively transferred mice were harvested five days after adoptive transfer. Spleens and lymph nodes were collected and mashed through a 70uM cell strainer. Tumors were minced and digested with Liberase TM (Roche) for 30 minutes and mashed through 70uM cell strainers. Isolated single cell suspensions were analyzed for Thy1.2⁺ CD8⁺ cells on a Galios Flow Cytometer (BD Coulter) and data was analyzed with FlowJo software (Treestar, Inc.)

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism Software using an unpaired, two-tailed Student's t-test, assuming equal population variances to determine the statistical significance between treatment groups. $p < 0.05$ was considered to be significant.

Results:

Sorafenib can be effectively combined with GM-CSF-secreting cellular immunotherapy in FVB/N mice

The therapeutic activity of Sorafenib was analyzed in the context of a GM-CSF-secreting, HER-2-overexpressing vaccine. NT2.5 tumor regression was accelerated in FVB/N mice that received the HER-2-specific vaccine in combination with Sorafenib relative to mice treated with either single agent Sorafenib, mock vaccine, or vehicle control alone (Figure 12A). Mice receiving combination therapy also showed improved tumor-free survival compared with mice receiving either single agent therapy (Figure 12B).

Sorafenib does not hinder vaccine-induced immune response

Next, the effect of Sorafenib on vaccine-induced immune responses was analyzed. Splenic CD8⁺ T cell IFN γ production in response to tumor and vaccine cell targets was used to assay vaccine-induced immunity. IFN γ production by splenic CD8⁺ T cells co-cultured with NT2.5 tumor cells or 3T3neu vaccine cells expressing B7.1 as targets was measured by ELISPOT. Sorafenib treatment alone did not induce a CD8⁺ T cell response to either NT2.5 tumor cells or vaccine cells; in contrast GM-CSF-secreting vaccination resulted in a robust CD8⁺ T cell response. Adding Sorafenib to vaccination did not inhibit the vaccine-induced CD8⁺ T cell response to HER-2-expressing target cells (3T3neuB7.1 or NT2.5B7.1, Figure 12C). Additionally, vaccinated mice receiving Sorafenib developed RNEU420-429-specific CD8⁺ T cell responses as well as mice that received vaccine alone (Figure 12D). These data show that Sorafenib does not interfere with vaccine-induced

immune responses and can be successfully combined with vaccination to enhance NT2.5 tumor clearance in immune competent FVB/N mice.

Sorafenib does not impede tumor infiltrating immune cells

The effect of Sorafenib treatment on the ability of immune cells to infiltrate the tumor was also analyzed. Tumors from vaccinated mice showed increased amounts of CD3⁺ T cells and CD68⁺ macrophages. Sorafenib treatment did not alter the infiltration of these cells into the tumor as similar numbers of each cell type were present in both treatment settings (Figure 13A and C). Treatment with vaccine alone or in combination with Sorafenib did not alter infiltrating Treg numbers relative to treatment control tumors. Staining for Ly6C/Ly6G, indicating the presence of granulocytic myeloid cells, was increased in mice that received vaccination in combination with Sorafenib.

Sorafenib increases HER-2-specific T cell accumulation in the tumor

To further evaluate the possible effect of Sorafenib therapy on the magnitude of the vaccine-induced locoregional T cell responses, adoptive T cell transfers with Clone 100 TCR transgenic T cells specific for RNEU420-429 were performed. Vaccination alone was sufficient to increase the number of adoptively transferred antigen-specific Thy1.2⁺ CD8⁺ T cells in the spleen and vaccine-draining lymph nodes relative to either mock vaccination or Sorafenib added to mock vaccination. HER-2 targeted vaccination also increased the number of T cells in the tumor relative to mock vaccination controls (Figure 14A-C). Combining Sorafenib with vaccine did not diminish the numbers of Thy1.2⁺ CD8⁺ cells found in the spleen and vaccine-draining lymph nodes (Figure 14A and B). Integrating Sorafenib with vaccination modestly increased the number of HER-2-specific T cells found

in the tumor (Figure 13C). These data suggest that Sorafenib may augment the ability of antigen-specific T cells generated by vaccination to accumulate in the tumor, thereby resulting in enhanced tumor clearance and tumor-free survival. Taken together, these data show that Sorafenib treatment may be effectively combined with HER-2 targeted DC-based vaccination to enhance tumor regression.

Figures:

Figure 12: Sorafenib can be effectively combined with vaccine in FVB/N mice. A, FVB/N mice (n=10) were tumor challenged on Day 0 and vaccinated and began daily Sorafenib or vehicle treatment on day 7 and followed for tumor growth and **B,** overall survival. **C,** FVB/N mice (n=10) were tumor challenged and at day 7 were vaccinated and began daily Sorafenib or vehicle treatment and 2 weeks post-vaccination, splenic CD8+ effector T cells were isolated and used for IFN γ ELISPOT with NT2.5B7.1 or 3T3neuB7.1 as targets or **D,** with p50 or NP peptide pulsed T2dq cells as targets. **, $P < 0.01$ and ***, $P < 0.001$.

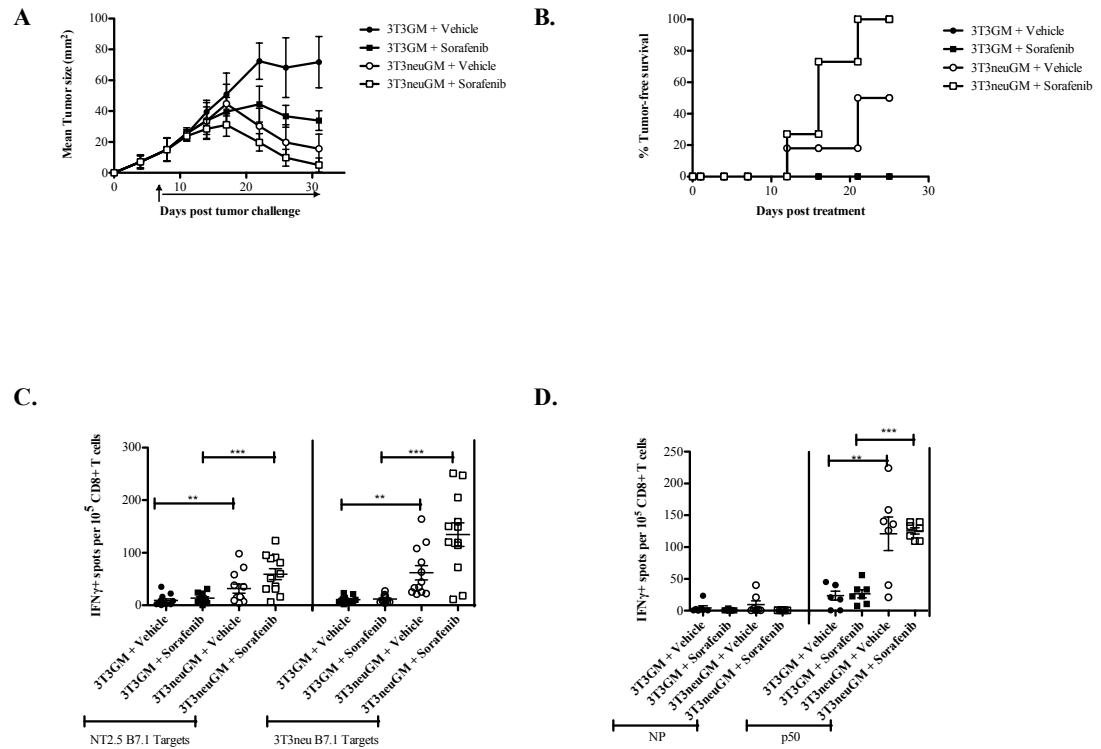
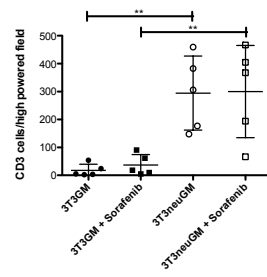
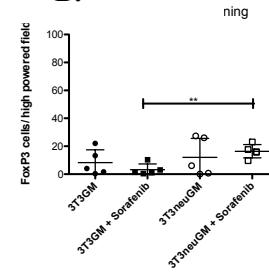


Figure 13: Sorafenib does not impede immune cell infiltration into the tumor. FVB/N mice (n=5) were tumor challenged on Day 0 and vaccinated and began daily Sorafenib or vehicle treatment on day 7. Tumors were prepared for histological examination 3 weeks after drug treatment. Immunohistochemistry to detect **A**, CD3, **B**, FoxP3, **C**, CD68, and **D**, Ly6C/Ly6G was performed at 20X magnification. Staining was quantified using Elements software. Graphs (mean + SD) are cell counts from 3-5 samples per group, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

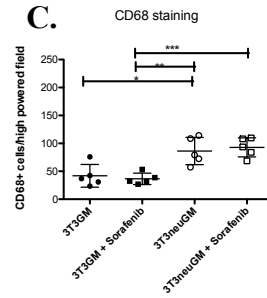
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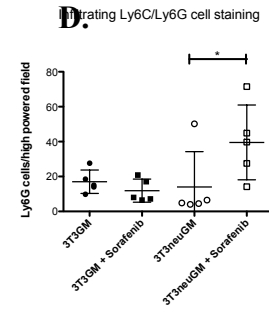
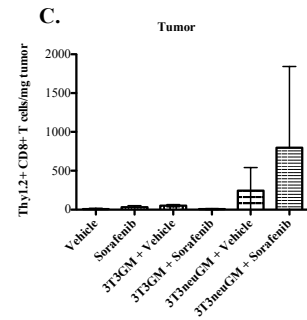
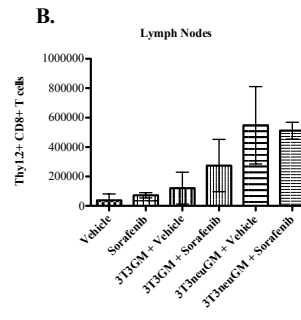
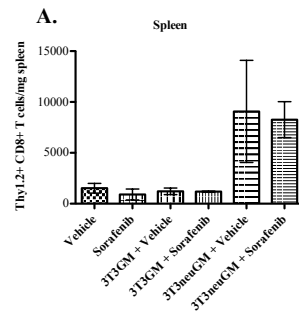


Figure 14: Sorafenib increases HER-2-specific T cells in the tumor. FVB/N mice (n=3) were tumor challenged and vaccinated and began daily Sorafenib treatment on day 7. Splenic CD8⁺ T cells were isolated from Clone100 mice and were adoptively transferred one day post-treatment. A, spleen, B, vaccine-draining lymph nodes and C, tumor were harvested 5 days post adoptive transfer and stained for antibodies specific for Thy1.2 and CD8. Samples were analyzed by flow cytometry, with the number of positive cells normalized to the tissue weight.



Conclusions:

The data presented here show two important new findings. First, the multi-kinase inhibitor of angiogenesis, Sorafenib, can be effectively combined with a DC-based vaccine in breast cancer. Second, combining vaccine with Sorafenib does not inhibit and may enhance vaccine-induced immunity. Although past studies have discouraged the use of Sorafenib in combination with immunotherapy, these studies add to the recently accumulating literature supporting partnering Sorafenib with immunotherapy. Given that single agent Sorafenib is often ineffective at producing lasting responses in breast cancer patients, these finding support repurposing Sorafenib in combination with immunotherapy as a new treatment avenue for breast cancer patients⁹.

The observed immune dependent mechanism for Sorafenib supports its use in combination with immune activating therapy such as vaccination. Therefore, the effect of combining Sorafenib with DC-based whole cell GM-CSF-secreting vaccine was examined. Many reports have discouraged the use of Sorafenib as a partner with immunotherapy as it has been found to inhibit DC function, reducing maturation and migration, and inhibit the production of OVA-specific CD8⁺ T cell responses *in vivo* in mice pre-treated with Sorafenib followed by peptide vaccination with adjuvant¹⁰. *In vitro* studies of lymphocytes from hepatocellular carcinoma patients demonstrated that pharmacologic doses of Sorafenib decreased effector T cell activation, whereas subpharmacologic doses selectively promoted the activation of effector T cells while blocking Treg function¹¹.

However, a growing body of evidence has since been published supporting the use of Sorafenib with combination immunotherapy. Sorafenib was shown to have a therapeutic

benefit in murine colon cancer when combined with MC38-CEA TRICOM vaccine¹². In an E.G7/OT-1 murine model of adoptive cell therapy with low dose Sorafenib, Sorafenib decreased the expression of immunosuppressive factors, and enhanced functions and migrations of transferred CD8⁺ T cells through inhibition of STAT3 and other immunosuppressive factors¹³.

The data reported here support the latter publications, demonstrating that incorporating Sorafenib with vaccine resulted in enhanced tumor clearance compared to single agent therapy. Additionally, vaccine-induced immune response was effectively maintained with the addition of Sorafenib. Sorafenib did not prevent vaccine-induced recruitment of CD3⁺ T cells to the tumor, suggesting that Sorafenib does not negatively affect the ability of T cells to gain access to the tumor to exert effector function. Finally, combining Sorafenib with vaccine increased the accumulation of adoptively transferred tumor-specific CD8⁺ T cells in the tumor with combination therapy than with either single agent. These data also support previous studies showing that angiogenesis inhibitors improve cellular immunotherapy¹⁴⁻¹⁶.

Sorafenib treatment combined with vaccine also increased Ly6G/Ly6C⁺ cell infiltrate. Enhanced tumor clearance as a combination therapy would suggest that these cells are not myeloid-derived suppressor cells, although this needs to be further examined. The increased in Ly6G/Ly6C staining may also suggest an increased neutrophilic infiltration in these tumors. Prior reports have shown enhanced T cell dependent, tumor-specific protective immunity as a result of increased Fas mediated-neutrophilic interactions with FasL expressing cells within the tumor^{17,18}. It is possible that Sorafenib may alter the expression of FasL, a known chemoattractant of Fas-expressing neutrophils, within the

tumor microenvironment. Therefore, Sorafenib could be promoting a local neutrophil-induced inflammatory response in the tumor based on the Fas/FasL interaction that when combined with increased antigen-specific T cells induced by vaccine, results in enhanced rate of tumor clearance. However, this still has to be investigated.

In conclusion, it is shown here for the first time, that Sorafenib can be effectively combined with DC-based, HER2-targeted cellular immunotherapy to enhance breast tumor clearance and improve tumor-free survival. Due to its ability to target multiple aspects of the tumor microenvironment, including host tumor cells, endothelial cells, and immune cells, further studies utilizing Sorafenib in combination with other immunomodulatory treatments, such as vaccination, are warranted.

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2013	Second Place Poster for Translational Research, Sydney Kimmel Comprehensive Cancer Center Breast Program
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2011	Runner Up Poster for Translational Research, Sydney Kimmel Comprehensive Cancer Center Breast Program

PUBLICATIONS

PEER REVIEWED PUBLICATIONS

1. **Erdinc Sunay MM**, Fox-Talbot K, Velidedeoglu E, Baldwin WM 3rd, Wasowska BA. Absence of FcγRIII results in increased proinflammatory response in FcγRIII-KO cardiac recipients. *Transplantation*. 2013 Oct 15;96(7):601-8.
2. **Sunay ME**, Marincola, F., Khleif, SN., Silverstein, SC., Fox, B, Galon, J and Emens, LA. Focus on the Target: The Tumor Microenvironment, Society for Immunotherapy of Cancer Annual Meeting Workshop, October 24th-25th 2012, *Journal for ImmunoTherapy of Cancer*. 2013 1:9
3. Asano H, Lee CY, Fox-Talbot K, Koh CM, **Erdinc MM**, Marschner S, Keil S, Goodrich RP, Baldwin WM 3rd. Treatment with Riboflavin and Ultraviolet Light Prevents Alloimmunization to Platelet Transfusions and Cardiac Transplants. *Transplantation*. 2007 Nov 15;84(9):1174-82.
4. Lee CY, Lotfi-Emran S, **Erdinc M**, Murata K, Velidedeoglu E, Fox-Talbot K, Liu J, Garyu J, Baldwin WM 3rd, Wasowska BA. The Involvement of FcR Mechanisms in Antibody-Mediated Rejection. *Transplantation*. 2007 Nov 27;84(10):1324-34.

ABSTRACTS

1. Neale, D. **Erdinc, M**, and Baldwin, WM 3rd. 275: Further evidence that trophoblast derived microparticles cause endothelial activation—A role in the pathogenesis of preeclampsia. SMFM abstract. American Journal of Obstetrics and Gynecology Volume 199, Issue 6, Supplement 1, December 2008, Page S88 *Society for Maternal-Fetal Medicine: 2009 29th Annual Meeting*

ORAL PRESENTATIONS

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|-----------|---|
| 5/21/2011 | Invited talk at Middle Atlantic Regional Meeting of the American Chemical Society, College Park, MD. “Can Anti-angiogenic tyrosine kinase inhibitors enhance cancer vaccines?” |
| 6/25/2010 | 3rd Annual Safeway Breast Cancer Retreat Mount Washington, MD. “Deciphering the Signaling Networks in the Breast Cancer Microenvironment.” |
| 6/14/2010 | Invited talk at Pathology Grand Rounds Johns Hopkins Hospital, Baltimore MD. “Identification of Novel Tumor Antigens by Vaccine-Induced Antibody Analysis in Patients with Metastatic Breast Cancer.” |

POSTERS

1. **Sunay, ME et al.** Sorafenib Modulates Macrophages to Promote T Helper Type 1 Immunity, *AACR Annual Meeting*, 2013.
2. **Sunay, ME et al.** Sorafenib Modulates Macrophages to Promote T Helper Type 1 Immunity, *Johns Hopkins Pathology Young Investigator’s Day*, 2013.
3. **Sunay, ME et al.** Mechanisms of Immune Modulation by Sorafenib in a HER2+ Breast Cancer Model, *Johns Hopkins Pathology Young Investigator’s Day*, 2012.

4. **Sunay, ME et al.** Elucidating Mechanisms of Immune Modulation by Sorafenib in Breast Cancer, *Johns Hopkins Pathobiology Training Program Annual Retreat*, 2012.
5. **Sunay, ME et al.** Understanding Mechanisms of Immune Modulation by Sorafenib in HER2+ Breast Cancer, *Johns Hopkins Sydney Kimmel Comprehensive Cancer Center Breast Program Retreat*, 2012.
6. **Sunay, ME et al.** Determining the Immune Mechanisms of Using Small Molecular Inhibitors to Improve the Efficacy of HER2/Neu Vaccination, *Johns Hopkins Pathology Young Investigator's Day*, 2011.
7. **Sunay, ME et al.** Immune Modulation by Sorafenib in a HER2+ Breast Cancer, *Johns Hopkins Pathobiology Training Program Annual Retreat*. Baltimore, MD, “
8. **Sunay, ME et al.** Mechanisms of Immune Modulation by Sorafenib in a Mouse HER2+ Breast Cancer Model, *Sydney Kimmel Comprehensive Cancer Center Breast Program Retreat*. 2011.
9. **Sunay, ME et al.** Determining Mechanisms of Immune Modulation by Sorafenib in a HER2+ Breast Cancer Model, *MARM ACS Annual Meeting*. 2011.
10. **Sunay, ME et al.** Identification of Novel Tumor Antigens by Vaccine-Induced Antibody Analysis in Patients with Metastatic Breast Cancer, *Sydney Kimmel Comprehensive Cancer Center Breast Program Retreat*, 2010.
11. **Erdinc, MM et al.** Vaccine-Induced Antibody Analysis in Patients with Metastatic Breast Cancer, *The Pathobiology Training Program Annual Retreat*, 2009.
12. **Erdinc, MM et al.** Identifying Novel Tumor Antigens by Vaccine-Induced Antibody Analysis, *Sydney Kimmel Comprehensive Cancer Center Breast Program Retreat*. 2009.